Salisphere development for salivary gland regeneration

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SALISPHERE DEVELOPMENT FOR SALIVARY GLAND REGENERATION

Thesis submitted for degree

DOCTOR of PHILOSOPHY

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SEPTEMBER 2016

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ABSTRACT

Salivary gland stem/progenitor cells are considered a promising solution for ameliorating salivary gland damage. mTOR signalling is known to play a role during salivary gland atrophy as ligation of the salivary main excretory duct results in the activation of mammalian target of rapamycin (mTOR). Cultured mouse and human salivary gland stem/progenitor cells *in vitro* are able to form spherical non-adherent clusters named salispheres. As mTOR plays a role during glandular atrophy, salispheres were cultured from un-operated, ligated and de-ligated glands. This project aimed to understand several factors on the culture of salispheres. Indeed, mTOR is inactivated in healthy glands but activated during atrophy. Measuring changes in mTOR activity in growing salispheres highlighted the importance of this network for salisphere survival, and a potential correlation between glandular atrophy and salisphere culture. Rapamycin treatment illustrated the necessity of mTOR for growing salispheres, whereas LiCl treatment suggested that GSK-3 inhibition stimulated the expansion of salispheres.

Interestingly, injury appeared to alter the growth behaviour of salispheres compared to controls. Mainly, salispheres adhere to a surface of the plastic dish and acquire fibroblastic-like structures suggesting that signalling alterations might be responsible for these changes. The detection of 4e-bp1 and S6 rp expression in salispheres suggested that mTOR was responsible for salisphere growth because it is involved in protein translation, but it was not responsible for the morphological modification post-injury. Changes in the ability of salispheres to adhere to a surface raised two questions. First, were morphological alterations mediated by the cytoskeletal rearrangement? For this reason, RhoA/ROCK and mTORC2 were investigated, as

they are both involved in the cytoskeletal organisation. The use of a ROCK inhibitor and torin1 to inhibit ROCK signalling and mTORC2 respectively, suggested that ROCK might played a role during atrophy as increased expression of p-FAK and CK5 expression in cultured salispheres from ligated glands and treated salispheres with ROCK inhibitor.

Second, was the neural input affected by injury and involved in the adherence of salispheres? *In vivo* and *in vitro* experiments of BoNT/A and neuropeptides were included to investigate if these treatments led to similar effects on salispheres as injury. However, only *in vivo* experiments of BoNT/A showed minor similarities, implying an unknown complex mechanism is responsible for these changes.

To translate the effect of the *in vitro* treatments on salivary glands, *in vivo* experiments were included. Injections of treated salispheres with several inhibitors were initially applied to normal salivary glands to determine their physiological response. Among the different inhibitors, LiCl not only showed significant effects on salispheres culture such as preventing salispheres from branching in collagen/matrigel culture and supporting their survival, but also showed an effect on glandular recovery during de-ligation. This suggested that LiCl might played a role in glandular recovery through salispheres. However, *in vivo* transplantation of ROCKi treated salispheres, which mimicked salispheres from ligated glands, had little or no effects on normal salivary glands. However, the activation of mTOR post ROCKi injection implies that ROCK signalling might be involved in the atrophic process.

In conclusion, my results show that mTOR an essential factor for salisphere growth and survival, and LiCl might be a promising tool for enhancing the recovery of glands

post-injury. Finally, the inhibition of RhoA/ROCK could be a factor associated with salivary gland atrophy, through ROCK associations with mTOR.

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to my first supervisor Dr. Guy Carpenter for his continuous support throughout my doctorate. I am grateful for the opportunities you have given me, for your patience, motivation and immense knowledge. I could not have asked for a better advisor and mentor. My sincere thanks also go to my second supervisor, Prof. Gordon Proctor whose support and guidance made it possible for me to carry out my research and made this PhD an enriching experience.

I would also like to thank all the members of Salivary Research Laboratory, who brought joy and humour to some long days. And a big thank you to Carl Hobbs, Mathew Arno and Martti Miamets for their support.

A special thanks to my family. Words cannot express how grateful I am to my mother and grandmother for always believing in me and encouraging me to follow my dreams. I would also like to thank all of my friends who supported me. In particular Hengi and Amy who had more faith in my ability than I did, and encouraged me all the way.

CONFERENCE PRESENTATIONS

- The role of mTOR for salispheres formation and development. 10th European Symposium on Saliva, Netherlands (2014).
- The effects of ROCK inhibition on salispheres morphology. The International Association for Dental Research, Croatia (2014).

TABLE OF ABBERVIATIONS

ANOVA	Analysis of Variance
AQP5	Aquaporin 5
AU	Artbitrary unit
BSA	Bovine serum albumin
DMEM-F12	Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12
DTT	Dithiothreitol
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
LiCl	Lithium chloride
NaCl	Sodium chloride
PBS	Phosphate buffered solution
SGK-1	Glucocorticoid-induced Protein Kinase 1
TBS-T	Tris-buffered saline Tween 20
TK	Tyrosine kinase
PI3K	Phosphotidylinositide 3-Kinase

1 INTRODUCTION

1.1 Salivary glands

Salivary glands are exocrine glands, which are present in the oral cavity. They are important as saliva hydrates and protect the mucosal surfaces, essential for the oral processing of food (Delporte and Steinfeld, 2006).

Salivary glands are classified according to size into major and minor glands, or according to their type of secretion: serous, mucous or mixed (seromucous). Major salivary glands include the parotid, submandibular and sublingual glands (Figure 1.1). Comparatively, there are approximately 500 to 1000 lobules of minor salivary gland tissue (Beale and Madani, 2006).

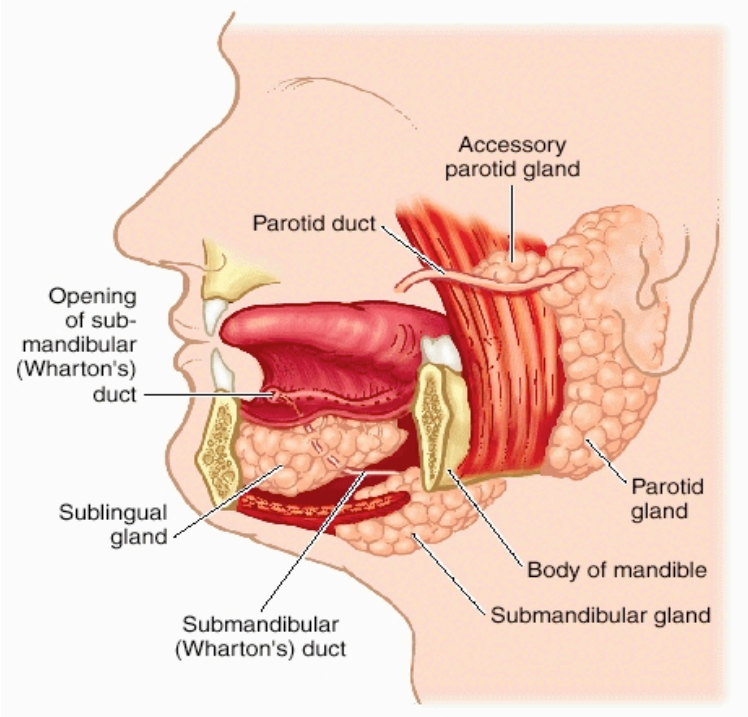


Figure 1.1 Anatomy of the major salivary glands (Dorland et al., 2007).

1.1.1 Parotid glands

The parotid glands are the largest salivary glands in humans. They are located over the ramous of the mandible and are surrounded by the sternocleidomastoid muscle. Parotid glands contain lymph nodes, facial nerves, the external carotid artery and the retromandibular vein. Saliva produced by the parotid glands is transported to the oral cavity by the Stensen's duct. The Stensen's duct is approximately 7 cm long and starts from the interior surface of the parotid gland, and ends at the upper second molar tooth (Beale and Madani, 2006).

1.1.2 Submandibular glands

The submandibular glands fill the major portion of the submandibular triangle, and comprise two lobes: the superficial and the deep lobe. Saliva produced by the submandibular glands is transported by its main duct (Wharton's duct), which lies in the floor of the mouth (Beale and Madani, 2006).

1.1.3 Sublingual glands

The sublingual glands are the smallest among the major salivary glands and they lie under the mucosa of the floor of the mouth. Sublingual glands comprise approximately 20 small ducts, which either drain directly into the floor of the mouth, or into the Wharton's duct (Beale and Madani, 2006).

1.1.4 Minor glands

Minor salivary glands exist in different locations in the oral cavity and are classified according to their location: buccal, labial, palatal, palatoglossal and lingual.

Additionally, they are considered mucous glands due to their mucous secretion (Berkovitz et al., 2009).

1.2 Mouse salivary glands

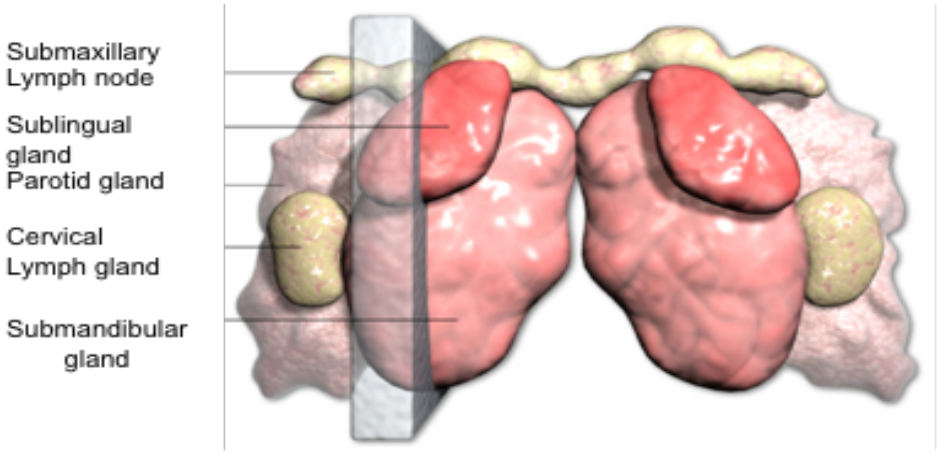


Figure 1.2 . Anatomy of major salivary glands in the mouse. Adapted from National Institute Health Environmental Health Sciences, 2011.

Salivary glands are composed of parenchyma and stroma. The parenchyma comprises the secretory and the ductal units, and the stroma the surrounding connective tissue. Within salivary glands are acinar cells, which first produce saliva, and ducts, for modifying and transporting saliva into the oral cavity (Figure 1.3) (Holsinger and Bui, 2006).

1.3 Histology

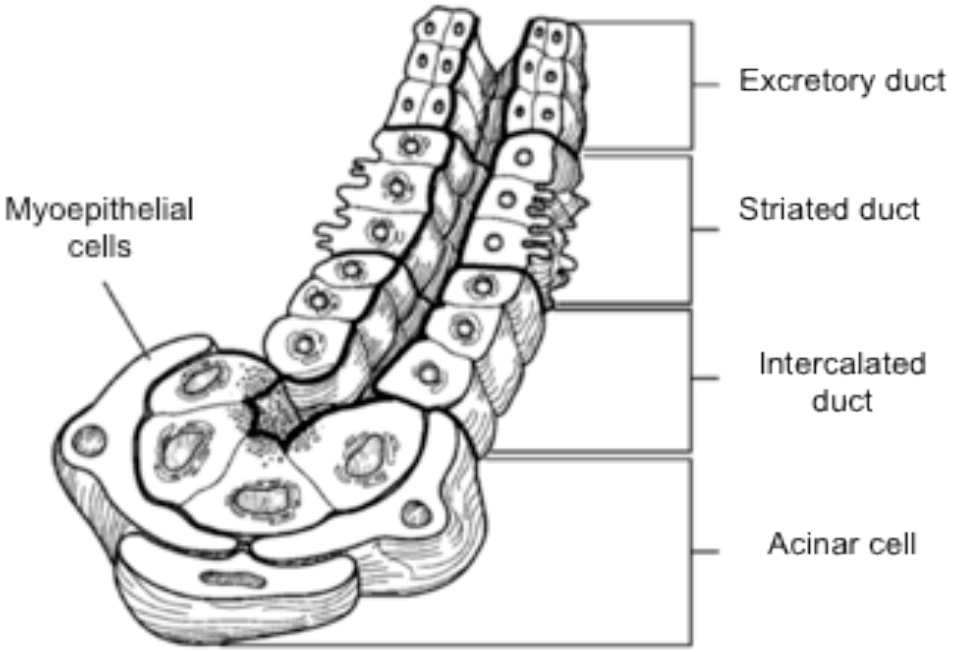


Figure 1.3 Microscopic anatomy of salivary glands. A salivary gland is composed of an acinar cell, which is surrounded by myoepithelial cells. The acinar cell is connected to an intercalated duct, followed by a striated duct and an excretory duct respectively. Adapted from Holsinger and Bui, 2006.

1.3.1 Acini

Acinar cells are classified into serous, mucous and seromucous. Serous acini produce a watery, protein-rich secretion, whereas mucous acini produce a viscous-rich saliva containing mucins (Holsinger and Bui, 2006). Acini are surrounded by myoepithelial cells, which facilitates saliva secretion (Humphrey and Williamson, 2001). Mucous cells produce mucins, which are considered large glycoproteins. The viscosity texture of saliva is due to the negative charge of mucins (Tucker, 2007).

1.3.2 Ductal system

Saliva produced by acinar cells is transported to the oral cavity via a ductal system, including the intercalated, striated and excretory ducts. The intercalated ducts are the first connected ducts to the acinar cells, followed by the striated ducts and the excretory ducts. The excretory ducts are the final duct network before saliva drains into the oral cavity (Edgar, 1992; Humphrey and Williamson, 2001).

1.4 Histology of mouse salivary glands

There are several microscopic differences between human and rodent salivary glands (Amano et al., 2012). For example, the ductal system in rodent contains granular convoluted ducts (GCT), which are located between the intercalated and the striated ducts. They produce and release hormones and different growth factors such as normal growth factor (NGF) and epidermal growth factor (Gresik, 1994; Amano et al., 2012).

Besides the morphological differences between human and rodent salivary glands, there is also sexual dimorphism. For example, the weight of a male submandibular gland is much higher than the weight of a female submandibular gland (Atkinson et al., 1959). Also, the GCT in a male submandibular gland is differentiated by the male testosterone hormone, whereas it is rarely developed in female submandibular gland (Amano et al., 2012, Gresik, 1994). Moreover, the level of aminotransferase activity in male submandibular glands is 10 times higher than in female submandibular glands (Hosoi et al., 1978).

1.5 Mouse salivary glands embryonic development

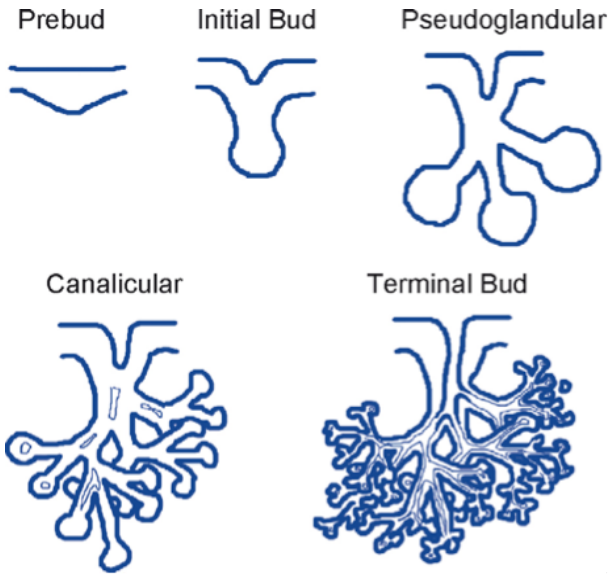


Figure 1.4 Salivary gland embryonic development. The stages of salivary gland embryonic development. Adapted from Tucker, 2007.

The embryonic development of mouse salivary glands begins at E11.5 where a thickening of the epithelium next to the tongue is observed; this stage is called the pre-bud stage (Figure 1.4). The process of thickening of the epithelium continues to the underlying mesenchyme producing a bud connected to the oral surface by a duct at E12.5. Then, this bud proceeds to the second, pseudoglandular stage, where the bud branches to produce a cluster of buds (Tucker, 2007).

Next, the process of branching morphogenesis begins at E13.5, where the epithelium produces four to five buds and a multi-lobed gland by E14.5. The ducts of salivary glands undergo cavitation at E15.5 and develop a lumen (Tucker, 2007). Apoptosis plays a role during the development of the lumen in the cavitation stage and occurs specifically in the ducts, and relies on caspase-3 as a mediator of the apoptotic pathway (Jaskoll and Melnick, 1999, Tucker, 2007). At stage E17 the

terminal bud stage is reached and a well–developed lumen is observed (Figure 1.4) (Tucker, 2007, Melnick and Jaskoll, 2000).

1.6 Factors affecting branching morphogenesis

The process of branching morphogenesis requires an interaction between the epithelium and the mesenchyme, where the mesenchyme controls the signals for this interaction (Tucker, 2007). There are several genes responsible for regulating each stage of branching morphogenesis. For example, *Fgf10* expression occurs during the pre-bud stage and the mutation of this gene prohibits the formation of salivary glands (Tucker, 2007; Ohuchi et al., 2000; Patel et al., 2006). Likewise, the *Pitx2* knockout mouse prevents salivary gland formation at E17.5 (Tucker, 2007, Szeto et al., 1999).

The branching process is regulated by the extracellular matrix (Rosenthal et al.), which is a continuous sheet of the basement membrane and separates the epithelium from the mesenchyme. There are several proteins that are present in the ECM such as glycosaminoglycans, fibronectin, collagen and integrins; each component plays a role in branching morphogenesis (Tucker, 2007). For instance, the inhibition of fibronectin blocks cleft formation (Sakai et al., 2003).

1.7 Saliva

1.7.1 Composition

Saliva contains many proteins, which are mostly produced by the salivary glands. These proteins differ among the glands, but some are produced by all glands, such

as immunoglobulin A, which is the main antibody of saliva. Additionally, salivary glands are rich with amylase, which thought to be important for post-mastication and clearing the mouth from food. In addition, proline-rich proteins (PRPs), comprised mostly of basic PRPs, are only present in the parotid glands, unlike the acidic PRPs that are present in both parotid and submandibular glands. Likewise, mucins are produced by the submandibular and sublingual glands, but are not produced by the parotid gland due to their serous secretion (Carpenter, 2013).

1.7.2 Functions

Saliva plays various roles in oral health such as digestion, lubrication and buffering, and possesses antimicrobial and antiviral properties (Carpenter, 2013; Amerongen and Veerman, 2002). Functions of saliva are dependent on the surface, that is teeth or mucosa, and can serve mutual or exclusive functions or both. The diagram below summarizes the functions of saliva according to the surface (Figure 1.5) (Carpenter, 2013; Melvin et al., 2005).

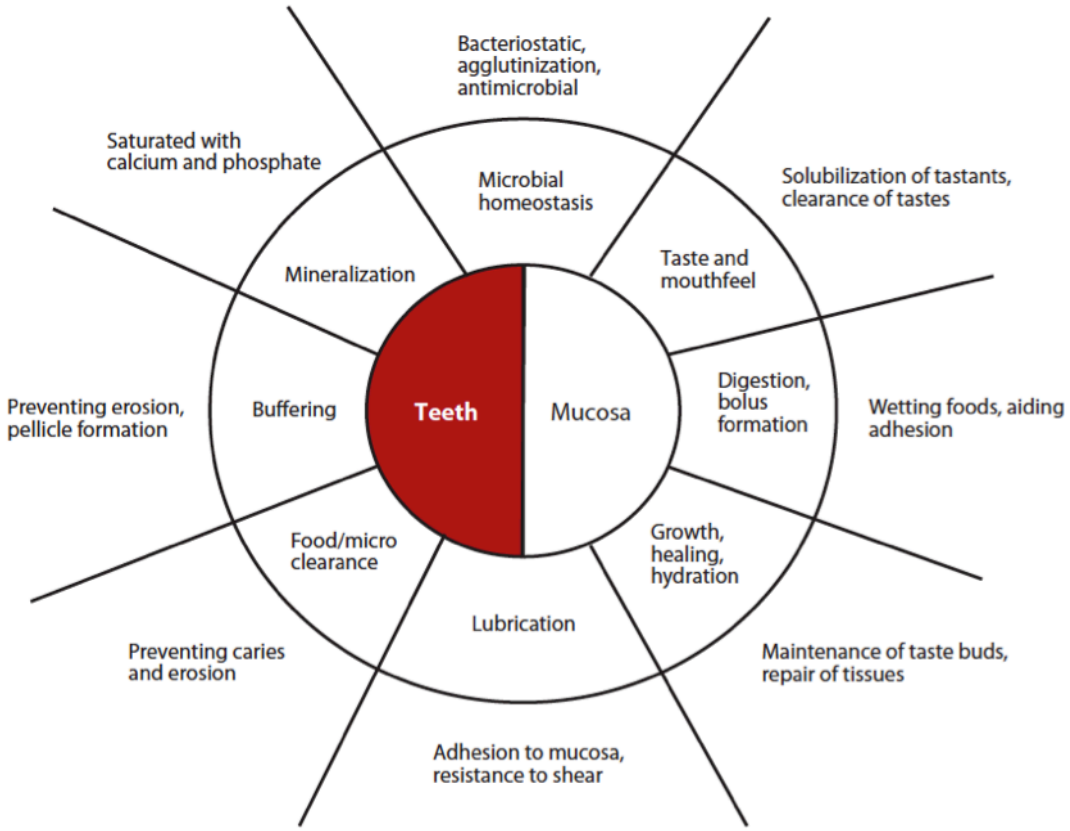


Figure 1.5 A summary of saliva functions depending on the surface [Adapted from (Carpenter, 2013)].

1.7.3 Saliva secretion

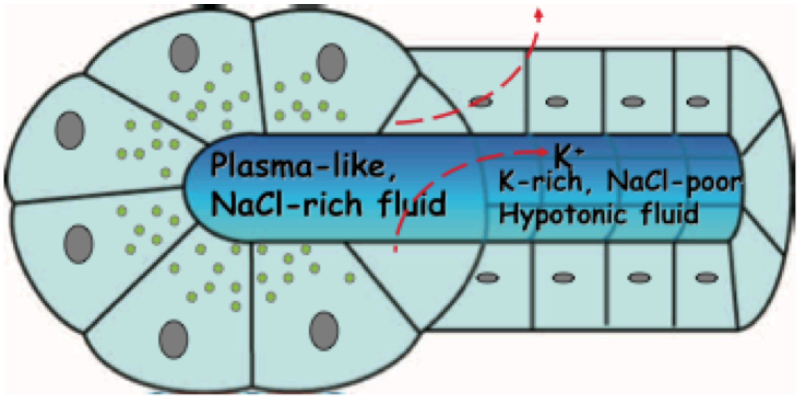


Figure 1.6 A simple model of salivary gland secretion [Adapted from (Catalan et al., 2009)].

In humans, most adult unstimulated salivary flow is around 0.5 mL/min^{-1}, while during sleep the salivary flow is around 0.1 mL/min^{-1} (Carpenter, 2013). The process of salivary secretion is summarized in two stages. First, an isotonic plasma-like primary secretion is produced, which is rich in sodium chloride. This primary saliva is modified while passing through the ducts, where the high levels of sodium chloride are reabsorbed and potassium is released (Figure 1.6) (Catalan et al., 2009; Melvin et al., 2005), producing a final hypotonic saliva (Humphrey and Williamson, 2001).

1.8 Autonomic nerve and salivary function

The secretion of saliva is nerve mediated; although, in some animals such as in sheep spontaneous nerve-independent saliva secretion. The parasympathetic nerves influence saliva secretion primarily by responding to afferent stimuli such as taste (Proctor and Carpenter, 2007). Parasympathetic stimulation is partly mediated by acetylcholine, while the sympathetic stimulation by noradrenaline (Garrett, 1987). The role of neural regulation of salivary secretion is explained further in Chapter 5.

1.9 Irreversible damage of salivary glands

There are several conditions that cause salivary gland hypofunction leading to oral dryness, a condition termed xerostomia, whereby saliva volume is decreased and saliva composition is altered (Nederfors, 2000; Saleh et al., 2015). Xerostomia causes difficulties in swallowing, chewing, impairment in taste, and increase dental caries (Sreebny and Valdini, 1987). These symptoms cause discomfort to patient and affect their quality of life. Some drugs are associated with salivary glands hypofunction, such as anti-HIV medication (Navazesh et al., 2009). In addition, systemic disorders such as Sjögren's syndrome and even radiotherapy treatment is

associated with xerostomia (Eisbruch et al., 200; Vissink et al., 2003; Saleh et al., 2015).

Radiotherapy is a common treatment for head and neck cancer. However, this treatment is one of the most common causes of salivary gland hypofunction (Deasy et al., 2010). Salivary glands are particularly susceptible to radiotherapy and undergo degeneration due to irradiation. However, the success of radiotherapy is often measured by the dose of radiation and the response of the patient to the treatment (Lin et al., 2008; Coppes et al., 2009; Dirix et al., 2008). For example, in animal studies radiation affects saliva flow rate, although the late effects vary among the parotid and the submandibular glands (Coppes et al., 2002).

In contrast to salivary gland atrophy, there are several conditions that contribute to hypersalivation. This condition usually is associated with neurological disorders such as Parkinson's disease and cerebral palsy in children, resulting in severe drooling (Seifert, 1987; Ellies et al., 2000).

1.10 Salivary gland regeneration

In normal conditions, salivary gland cells undergo autologous cell division for tissue homeostasis and damage repair. However, in the case of injury, normal autologous cell division is insufficient for maintaining tissue function and repairing the damage. Hence, progenitor cells are thought to be important for salivary glands regeneration. There are two common models used for studying regeneration, which are described below (Okumura et al., 2012).

1.10.1 Ligation/de-ligation

Ligation is a method used to understand the importance of salivary gland progenitor cells and the origin of new cells undergoing regeneration (Okumura et al., 2003; Cotroneo et al., 2010). Ligation of the main excretory duct induces apoptosis and causes the depletion of acinar cells, followed by ductal cell proliferation (Sato et al., 2007). While de-ligation has shown the ability of the salivary gland to recover and produce mature acinar cells (Takahashi et al., 1998). De-ligation causes the upregulation of the neonatal protein SMG-B and the recovery of Aquaporin 5 (AQP-5) which is usually expressed in the intercalated ducts of mature glands and in the acinar cells during the neonatal development (Cotroneo et al., 2010).

1.10.2 Radiation

Radiation is another model for studying regeneration and characterizing progenitor cells of salivary glands. Irradiation of salivary glands has either acute or chronic effects on salivary function, where the dose of radiation plays a role in the gland recovery. The use of radiation as a model helps to determine the number of remaining progenitor cells post-radiation, therefore assessing the regenerative capacity (Pringle et al., 2013). In addition, radiation studies help to elucidate the role of primitive cell transplantation into damaged gland tissue during regeneration (Feng et al., 2009). Although radiation is a well-used model, for the purpose of this thesis the ligation/de-ligation model was used.

1.11 Salivary gland stem/progenitor cells

Stem cells are defined as undifferentiated cells that are capable of differentiating into any specific tissue type and have a high degree of self-renewal. They play a role in homeostasis and are involved in tissue regeneration of many organs (Bishop et al., 2002; Korbling and Estrov, 2003). Oral tissue is believed to have a high number of stem cells (Egusa et al., 2012); although, a true stem cell has yet to be found in salivary glands. A true stem cell is characterized by the unlimited self-renewal and is considered the precursor of progenitor cells. Due to the lack of universal markers it is difficult to identify and purify stem cells (Kimoto et al., 2008). Label-retaining studies explain the process of asymmetric division of stem cells in tissue development and regeneration. A stem cell can produce two daughter cells: one stem cell and one transient cell (Kimoto et al., 2008; Coppes et al., 2009).

Progenitor cells are more lineage committed and organ-specific. They also have less capacity to self-renew as they become more differentiated to produce one cell type. Importantly, a stem cell might produce a differentiated daughter cell, which can undergo further cell division and produce progenitor cells. This process results in the tissue homeostasis, through their differentiation and maintenance of the stem/progenitor pool (Lombaert and Hoffman, 2010; Lombaert et al., 2011; Pringle et al., 2013).

A true stem cell is able to produce all type of cells of specific organ such as the mammary gland. However, the identification of a single stem cell that can produce the whole epithelial cell types is still unknown (Lombaert et al., 2011, Stingl et al., 2006).

1.12 Localization of salivary gland stem/progenitor cells

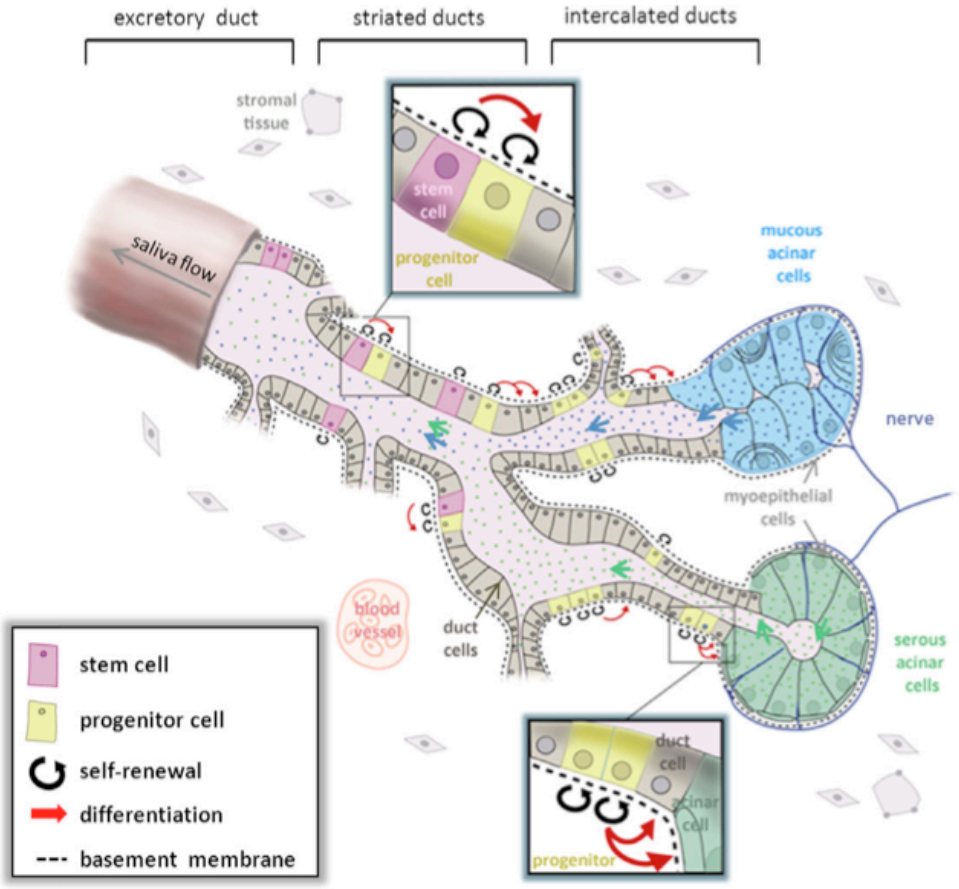


Figure 1.7 The location of stem and progenitor cells in salivary glands. It has been suggested that primitive cells are localized in the main excretory and striated ducts, while the progenitor cells are localized in the striated and intercalated ducts. This stem cell pool is believed to be responsible for replacing and producing mature acinar cells and duct cells [Adapted from (Pringle et al., 2013)].

Label retaining cells (LRCs) studies have localized the stem/progenitor cells in the ductal compartment (Denny et al., 1993; Man et al., 2001; Kimoto et al., 2008). In male rats, cell labelling suggested the differentiation of intercalated ducts and striated ducts into granular ducts (Denny et al., 1993). The pattern of labelled cells changes with time suggesting that cells move from the intercalated ducts to the granular ducts or striated ducts as well as to the acini, resulting in cell differentiation (Figure 1.7) (Zajicek et al., 1985). However, the number of labelled intercalated ducts

decreases with time. These intercalated cells have shown to differentiate into both acinar and granular duct cells (Man et al., 2001). The use of LRCs helped in identifying a small pool of stem/progenitor cells of salivary glands. Ligation/de-ligation experiments on rat submandibular glands illustrated the existence of LRCs during regeneration (Kimoto et al., 2008). Although it is believed that irradiation sterilizes the residing progenitor cells, previous studies have shown that LRCs in salivary glands are maintained post-radiation and these cells are considered progenitor cells (Konings et al., 2005; Chibly et al., 2014).

Human and mouse salivary gland stem/progenitor cells are thought to be located in the ductal compartment (Sato et al., 2007; Lombaert and Hoffman, 2010). Previous work suggested that stem cells are responsible for supplying the progenitor cell pool and are also involved in replenishing duct and acinar cells (Pringle et al., 2013).

1.13 Salivary gland stem/progenitor cell expressing markers

The identification of stem/progenitor cells by specific protein markers is essential for their phenotypic validation. However, isolating stem/progenitor cells from solid tissues, such as salivary glands, requires dispersing tissue into cell suspension by mechanical forces and enzymatic digestion (Coppes and Stokman, 2011).

There are different markers that are important for assessing salivary gland regeneration such as CD49f, Sca-1, c-kit and Ascl3. The expression of stem-associated markers in the ducts indicates the presence of these progenitor cells (Rugel-Stahl et al., 2012; Nanduri et al., 2013).

it is one of the important stem cell markers (Edling and Hallberg, 2007). Studies on c-kit expressing cells have shown the capacity of these cells to regenerate and restore salivary gland function (Lombaert et al., 2011). For example, transplanting c-it^+ cells into an irradiated gland resulted in the production of differentiated acinar cells and progenitor cells (Nanduri et al., 2013).

These stem cell markers are used to assess the *in vitro* differentiation and *in vivo* function (Pringle et al., 2013). For instance, c-kit expressing cells in mouse and human have been tested for *in vitro* differentiation, but only mouse expressing c-kit have been tested for the *in vivo* function (Lombaert et al., 2008a; Feng et al., 2009, Nanduri et al., 2011).

In addition, the transcription factor Ascl3 was also established as a marker for progenitor cells in salivary glands. Ascl3 expressing cells are localized in all mature ducts and only represent a subset of progenitor cell population (Bullard et al., 2008).

The identification of these markers is important to identify the precursor of these cells, which are involved in maintenance and regeneration.

1.14 Salivary gland stem/progenitor cells and regeneration

The irreversible damage of salivary gland function by irradiation is associated with the sterilization of primitive glandular stem cells (Konings et al., 2005). Salivary glands are highly radiosensitive as severe loss of saliva production is observed by phase 1 and phase 2 (Coppes et al., 2001). A potential treatment is bone-marrow derived cell (BMC) transplantation to regenerate damaged salivary glands (Lombaert et al., 2006a; Sumita et al., 2011). However, the transplantation of salivary gland stem/progenitor cells may be more beneficial, because *in vivo* studies have shown a native supply of stem/progenitor cells in salivary glands (Denny et al.,1993; Man et al., 2001; Lombaert et al., 2008a).

Additionally, mouse salivary gland Sca-1^+/c-kit^+ expressing cells are able to differentiate into pancreas and liver lineages (Hisatomi et al., 2004). The use of stem cell markers has demonstrated that as few as 300 c-kit expressing cells can recover salivary gland function (Lombaert et al., 2008a; Nanduri et al., 2011). In addition, a functional recovery of damaged salivary glands was observed by transplanting around 5,000 of CD29, CD49f or CD133 expressing cells (Nanduri et al., 2011). Parasympathetic nerves are also important for regeneration, since the parasympathetic nerves are essential for maintaining $K5^+$ epithelial progenitor cells during development (Knox et al., 2010). In the context of regeneration, a reduction of parasympathetic innervation resulted in a loss of acinar cells. This suggests that maintaining parasympathetic innervation after injury is important for epithelial tissue regeneration (Knox et al., 2013).

Although no *in vivo* functional analysis has tested human tissue, there are several studies that have shown the ability of human primary salivary gland cells to differentiate *in vitro*. Either using a hydrogel culture system or a matrigel-coated matrix, primary human salivary gland cells displayed some aspects of differentiation (Joraku et al., 2007; Feng et al., 2009; Maria et al., 2011, Pradhan-Bhatt et al., 2013). A three-dimensional culture system has also been used to determine their ability to grow and differentiate into a functional salivary acinar-like structure (Feng et al., 2009; Pradhan-Bhatt et al., 2013).

Recently, the xenotransplantation model has also indicated the therapeutic potential and capacity of human salivary gland stem/progenitor cells to restore salivary function *in vivo* (Pringle et al., 2016). Although, it is important to understand and consider the biological differences between humans and rodents and their responses to radiation (Lombaert et al., 2017).

1.15 Salispheres

The isolation and culture of salivary gland stem/progenitor cells typically involves using a monolayer or non-adherent culture system (Pringle et al., 2013). A monolayer culture system was mostly performed from rat salivary glands, while a non-adherent culture system was performed from mouse salivary glands (Kishi et al., 2006; Lombaert et al., 2008a; Nanduri et al., 2011; Neumann et al., 2012).

Advances studies using glandular stem cells, for example, cells derived from the mammary gland have applied the non-adherent technique (Reynolds and Weiss, 1992; Liao et al., 2007). This method is useful for driving a functional group of progenitor cells (Pringle et al., 2013). Likewise, non-adherent culture of salivary

gland stem/progenitor cells was developed successfully (Lombaert et al., 2008a; Nanduri et al., 2011; Pringle et al., 2011).

Isolation and culture of salivary gland stem/progenitor cells *in vitro* grow in spherical, non-adherent clusters named as salispheres (Lombaert et al., 2008a; Coppes et al., 2009; Nanduri et al., 2011). This process initially requires dispersing the tissue by enzymatic digestion and mechanical forces. This results in a mixed population of dead and living cells where dead cells are separated and stem/progenitor cells are enriched during the intermediate step of cell culture (Coppes and Stokman, 2011).

These spheres appear around day two or three after culturing in serum-free medium containing growth nutrients. Salisphere formation is similar for both human and mouse cells (Lombaert et al., 2008a; Feng et al., 2009). Interestingly, salispheres from human and mice express c-kit, and their development into a spherical formation allows them to differentiate *in vitro* (Sato et al., 2007; Pringle et al., 2013).

1.16 Role of signalling pathways in salivary gland regeneration

Growth factors and signalling networks play an important role in salivary gland stem/progenitor pool regeneration (Lombaert et el., 2008b; Silver et al., 2010). Several studies have been performed to understand the role of signalling networks on salivary gland regeneration such as Wnt signaling (Hai et al., 2010).

Studies on systemic stimulation of growth factors have shown a role in rescuing salivary glands against irradiation-induced damage. For example, intravenous injection of insulin-like growth factor 1 (IGF-1) replenished apoptotic cells caused by radiation and preserved salivary gland function via the activation of Akt (Limesand et

al., 2009). In addition, keratinocyte growth factor (KGF) also increases salivary gland progenitor cell population and has a role in improving salivary gland function post-radiation (Lombaert et al., 2008b). Moreover, a study by Xiao and colleagues (2013) suggests that aldehyde dehydrogenase-3 activator (ALDH) may protect acinar cells against radiation and increase saliva production.

Gene expression analysis showed that glial cell line-derived neurotrophic factor (GDNF) is upregulated post-radiation in mice submandibular glands. The administration of GDNF improved saliva production and enrichment of functional acinar cells in irradiated glands (Xiao et al., 2014).

Several studies have also assessed the role of protective intrinsic signalling pathways in salivary glands. For instance, the activation of hedgehog (Hh) exhibited a role in protecting salivary gland stem/progenitor cell and parasympathetic innervation against irradiation (Hai et al., 2014).

Wnt signalling is activated in stem/progenitor cells compartment, which decreases parallel to age of mice. However, a significant upregulation of Wnt activity accompanied with Hh signalling occurs as a consequence of main duct ligation.

This suggests that the activation of Wnt and Hh are responsible for the molecular control of the regenerative process (Hai et al., 2010). In previous studies, the concurrent activation of Wnt signalling protected salivary gland function against a single dose of irradiation and the concurrent activation of the Wnt pathway inhibited apoptosis, and protected the stem/progenitor pool (Hai et al., 2012). Wnt also plays a role in the long-term expansion of salivary gland stem/progenitor cells; for example,

the transplantation of Wnt-induced stem/progenitor cells into a damaged gland resulted in the recovery of tissue homeostasis (Maimets et al., 2016).

The mammalian target of rapamycin (mTOR) is another important pathway for cell growth and differentiation. It is considered an important regulator of skeletal muscle hypertrophy and atrophy (Bodine et al., 2001). In normal adult salivary glands, mTOR is inactive; however, it has been demonstrated that mTOR may play a role during salivary gland injury as it protects a subpopulation of acinar cells (Silver et al., 2010). The administration of rapamycin which is an inhibitor of mTOR can delay ligation-induced atrophy. The full inhibition of mTOR suggested that rapamycin prevents the degradation of secretory glycoproteins by preserving the mucin content post-treatment (Bozorgi et al., 2014). Since mTOR is an important signalling network for cell growth, and most importantly, is associated with salivary gland atrophy, the reminder of this chapter will focus on the characteristics of mTOR.

1.17 mTOR

mTOR plays a significant role in growth and cell proliferation (Sarbassov et al., 2005). The TOR protein is a serine threonine kinase, which targets the compound rapamycin. It interacts with several proteins to produce two main complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante and Sabatini, 2012a).

1.17.1 mTOR general structure

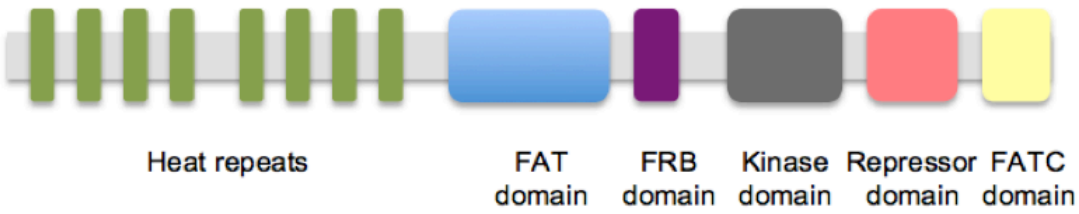


Figure 1.8 mTOR domain structure.

mTOR is composed of 2549 amino and comprises several conserved domains. The first 1200 amino acids are tandem HEAT repeats, which stands for huningtin, elongation factor 3, a subunit of PP 2A and TOR (Figure 1.8) (Gingras et al., 2001b). A FAT (FRAP, ATM, TRRAP) domain is located after the HEAT repeats and binds to the FKPB12-rapamycin binding (FRB) domain (Brown et al., 1994). A kinase domain (KB), a repressor domain and FAT carboxyl-terminal (FATC) domain are located after the FRB domain and regulate mTOR activity (Perry and Kleckner, 2003).

1.17.2 mTORC1

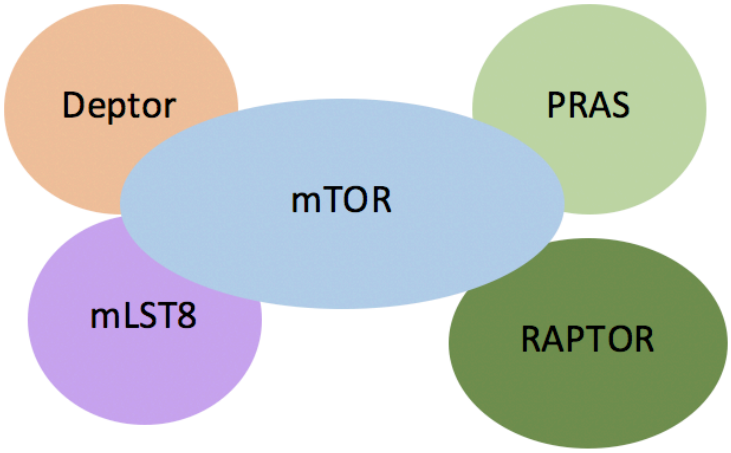


Figure 1.9 The main components of mTORC1.

mTORC1 consists of five components: the catalytic subunit which is mTOR, a regulatory-associated protein of mTOR (Raptor), mammalian lethal which hSec13 protein 8 (mLST8 or GβL), proline rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (Deptor) (Laplante and Sabatini, 2009). There are several upstream signals that regulate and control mTORC1 activity such as growth factors, energy levels and amino acids (Laplante and Sabatini, 2012a).

1.17.3 mTORC2

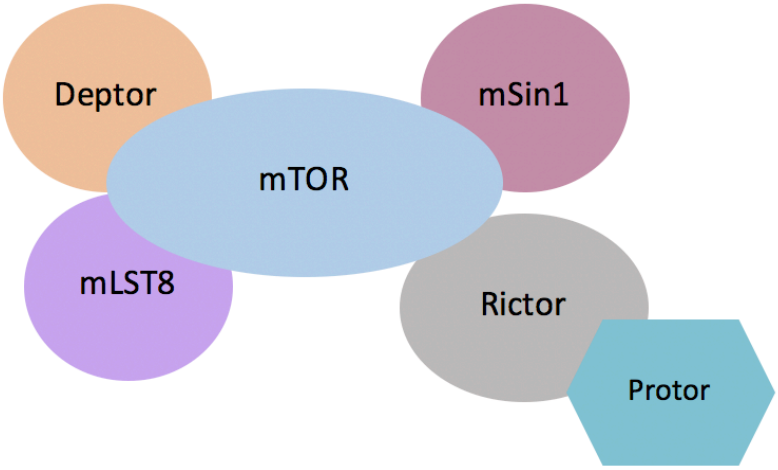


Figure 1.10 The main components of mTORC2.

The second complex of mTOR is composed of six components, where it shares some of mTORC1 proteins such as Deptor and mLST8. It also contains rapamycin insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1) (Laplante and Sabatini, 2009).

1.17.4 mTOR mechanism of action

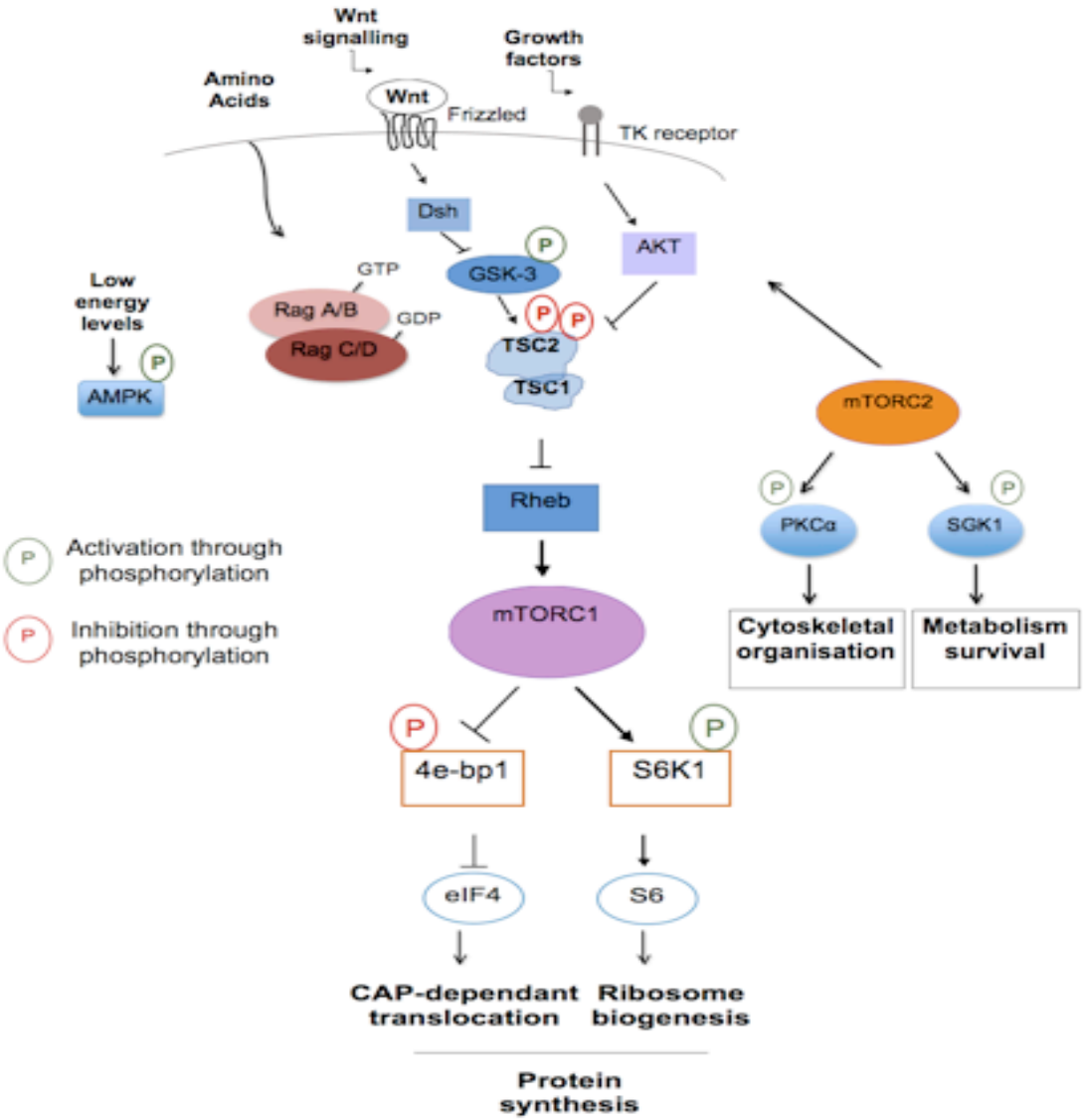


Figure 1.11 A schematic diagram for mTOR signalling pathway. Several factors play a role in the activation of mTOR signalling such as energy levels, amino acids, Wnt signaling and growth factors. The activation of Wnt signalling by the binding of Wnt protein to the frizzled receptor leads to the phosphorylation of GSK-3 by Dishevelld and the activation of mTOR. Growth factors also play a role in mTOR activation through Akt, which phosphorylates TSC2 subsequently activating mTOR via Rheb. As a result of mTOR activation, the main substrates of mTOR, 4e-bp1 and S6K, are phosphorylated. Various processes are regulated by mTOR such as protein synthesis and autophagy.

The mechanisms of action of the two complexes of mTOR are different in terms of their upstream regulators and downstream effectors. In the case of mTORC1, there are several upstream regulators, including growth factors, energy levels, stress, amino acids and Wnt signalling (Inoki et al., 2006; Sengupta et al., 2010; Laplante and Sabatini, 2012b; Jewell et al., 2013). The heterodimer, tuberous sclerosis 1 or hamaratin (TSC1) and tuberin (TSC2), cooperate in mTORC1 activation (Inoki et al., 2003b). The activation of mTOR by growth factors and Wnt signals subsequently phosphorylates TSC2, which in turn inhibits Ras homolog enriched in brain Rheb and thus activates mTOR (Figure 1.11) (Inoki et al., 2006; Sengupta et al., 2010). Rheb is a small GTPase which has been shown to be regulated by TSC2 and involves in mTOR activation. mTORC2 regulates several processes such as protein synthesis, lipid synthesis and autophagy via phosphorylation of two main substrates, 4e-bp1 and S6 kinase (S6K) (Jung et al., 2010; Laplante and Sabatini, 2012b; Lloyd, 2013). The expression of 4e-bp1 and S6K subsequently leads to the phosphorylation of eIF4 and S6 respectively; thus, regulating cell growth and protein synthesis (Jastrzebski et al., 2007; Magnuson et al., 2012). There are three isoforms of 4e-bp1 phosphorylation, which include α (hypo-phosphorylated), β (phosphorylated) and γ (hyper-phosphorylated). The function of eIF4 is dependent on the degree of 4e-bp1 phosphorylation (Beretta et al., 1996; Gingras et al., 2001a; Silver et al., 2010).

Although mTORC2 is not as well understood as mTORC1, mTORC2 it regulates several elements such as Akt which is involved in apoptosis, metabolism and proliferation. mTORC2 also regulates glucocorticoid-induced protein kinase 1 (GSK1), which is responsible of ion transport and growth. Also, protein kinase C-α (PKC-α), which regulates cell shape (Garcia-Martinez and Alessi, 2008; Laplante and Sabatini, 2012b).

1.17.5 Rapamycin

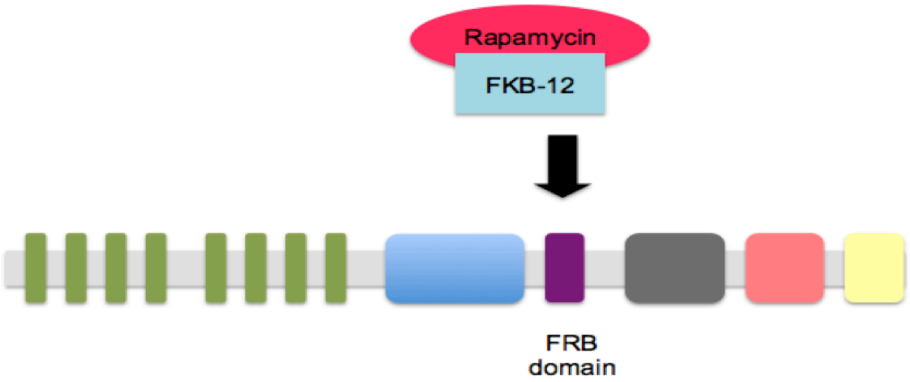


Figure 1.12 Rapamycin cite of action on mTORC1

Rapamycin is an anti-fungal molecule, which was isolated from the soil in the Eastern Islands in the 1970s. It is produced by the bacterial species *Streptomyces hygroscopicus* (Vezina et al., 1975). Rapamycin is also considered a potent molecule for inhibiting cell proliferation and is a valuable molecule for studying cell growth (Laplante and Sabatini, 2012a). The ability of rapamycin to bind to the FRB domain results in the inhibition of mTORC1 but not mTORC2 (Jacinto et al., 2004; Ballou and Lin, 2008). Rapamycin is also associated with autophagy and characterized by an anti-proliferative property, which could benefit patients with cancer (Hidalgo and Rowinsky, 2000; Cai and Yan, 2013).

1.18 Aim

The aim of this project was to explore factors affecting salispheres culture during normal development and during injury. Because the mTOR pathway is an essential signalling network responsible for cell cycle progression and it is activated in atrophic salivary glands, I focused on the role of mTOR in the development of salispheres from normal and injured glands. My aim was to investigate if mTOR is fundamental for salispheres survival. I also assessed the morphological differences and mTOR status between cultured salispheres from normal and injured glands, to determine whether mTOR is associated with injurious morphological modifications. In addition, I investigated possible factors associated with injury such as cytoskeletal rearrangements and neural influences. *In vivo* transplantation of treated salispheres with the different inhibitors into mouse salivary gland will assist in translating if the inhibitors could help in glandular recovery or associated during atrophy.

2 MATERIALS AND METHODS

2.1 Animals

Adult female ICR mice were obtained from Charles River Laboratories (Margate, UK) weighing 25-30 g upon arrival. Mice were housed in groups of four, supplied with food and water *ad libitum*. A 12 h light-dark cycle was maintained at constant temperature of 20-22°C, and environmental enrichments (tunnel and nesting material) were provided in each cage. Animals were kept for one week under clean conditions to allow them to acclimatize to their new environment before beginning any experimental procedure. The mice were terminally anesthetized by an overdose injection of pentobarbitone. Submandibular glands were dissected and all procedures were carried out under aseptic conditions. All animal procedures were conducted under the UK Home Office Animal (Scientific Procedures) Act 1986.

2.2 Salivary gland stem/progenitor cell isolation and culture

Salivary gland stem/progenitor cells were isolated and cultured according to an established protocol (Pringle et al., 2011).

Dissected submandibular glands were chopped for around 3 minutes. The homogenate was digested for 20 minutes using a mixture of collagenase II (23 mg/ml), hyaluronidase (40 mg/ml) and $CaCl_2$ (50 mM) for 80 mg gland weight in 5% BSA HBSS buffer. Then, the homogenate was mixed by pipetting and incubated for 20 minutes. The digestion and mixing steps were repeated with gentle mechanical movement at 37°C. Next the homogenate was washed twice with 5% BSA HBSS buffer. Following washing, the homogenate was filtered by 100 μm cell strainer then through a 50 μm pore size filters and a 25G needle. Pelleted cells were re-suspended in SC medium which contains DMEM/F-12, penicillin/streptomycin (1%),

glutamax (1%), EGF (20 ng/ml), FGF (20 ng/ml), N2 supplement (1/100), insulin (10 µg/ml) and dexamethasone (1 µM) then plated in non-coated 12-well dishes. All pelleting steps were performed at 400 g for 5 minutes.

All reagents were purchased from Sigma-Aldrich (Dorset, UK), except HBSS, glutamax, collagenase II and N2 supplement, which were purchased from GIBCO (Thermo Fisher Scientific Inc., Carlsbad, CA).

2.3 Cell/salispheres counting

For counting, the Bio-Rad TC20 automated cell counter (Bio-Rad Laboratories Ltd, Hertfordshire, UK) was used for counting cells prior culture (at day 0). The cell pellet was re-suspended in 1 ml of SC medium and 10 µl of cells were mixed with 10 µl of trypan blue (Thermo Fisher Scientific Inc., Carlsbad, CA) and loaded onto a slide for reading. The number of salispheres was determined by counting 10 µl, then the number of salispheres in 1 ml volume of media was calculated according to the equation below:

Total number of salispheres / ml = number of salispheres x 1000 µl of media / 10 µl

2.4 Imaging

Salispheres were imaged at different magnification including 10x, 20x and 40x and at different time-points depending on the experimental design by the phase-contrast microscopy (Olympus Essex, UK).

2.5 Size of salispheres

The size of salispheres was determined by measuring the area of 5-10 salispheres at 40x magnification and averaged by Image J software version 1.46 from each individual experiment (NH Maryland, MD, USA).

2.6 Salispheres collection

Salispheres were collected for protein analysis by pelleting the spheres at 3000 rpm for 5 minutes. The pellet was washed with PBS and lysed by RIPA buffer and stored at -20°C for further analysis.

2.7 Gel electrophoresis and Immunoblotting

Samples were processed for SDS-PAGE gel electrophoresis under reducing conditions. The samples were prepared with 1/4 LDS and 1/10 DTT of total sample volume. Protein separation was carried out either by loading equal protein concentrations or equal volumes of sample into NUPAGE® 4-12% Bris-Tris gel (Life technologies, Paisley, UK) for 32 minutes at 200 V and 125 mA. The reason behind using equal volumes of cells/salispheres homogenates rather than equal protein concentrations was to maximize concentration of loaded proteins in all lanes. Co-staining of blots with β-actin or GAPDH allowed normalization between lanes. Gels

were either used for Periodic Acid Schiff's staining and Coomassie Brilliant Blue staining, which are explained below in sections 2.15 and 2.16, or proceeded to the electroblotting step.

Proteins were electroblotted onto 0.45 µm nitrocellulose membranes (Aderman and Co., Kingston-Upon-Thames, UK) for one hour at 30 V and 200 mA. Transferred proteins on the nitrocellulose membranes were then processed for immunoprobing. The membrane was blocked with Tris-buffered saline tween 20 (TBST) (20 mM TRIS, 150 mM NaCl, 0.1 % Tween-20, PH 7.6) for one hour followed by either an hour incubation or overnight incubation with primary antibody. Next, the membrane was washed three times for 5 minutes each with TBST. The membrane was incubated for two hours with secondary antibody and was washed 5 minutes, three times for 5 minutes with TBST, three times with TBST and visualized using the ChemiDoc imaging system (Bio-Rad Laboratories, Hertfordshire, UK) after adding the ECL substrate (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Equal volumes of the reagents were mixed and applied to the membrane for visualizing protein expression. All of the running buffers, transfer and gels were purchased from Novex (Invitrogen, Carlsbad, CA). The function of ChemiDoc imaging system is mainly based on measuring the band intensities of protein of interest where the band intensity increases parallel to time of exposure (Figure 2.1). The pattern of protein expression is similar regardless if the band intensity was measured at 5 seconds or 60 seconds (Figure 2.2). However, all immunoblots have been chosen before 40 seconds as the blots become saturated after 40 seconds. Appendix B is a supplementary of five concentrations of submandibular gland tissue homogenates (Table 2.2) which have been probed with β-actin at different time-points to illustrate the principle of ChemiDoc system.

2.8 Antibodies

Antibodies	Abbreviation	Molecular weight (kDa)	Dilution	Company
Phospho 4ebp1	p-4e-bp1	~17	1:1000	Ce;; Signaling New England Biolabs
Phospho S6 ribosomal protein	p-S6 rp	32	1:1000	Cell Signalling, New England Biolabs
Beta actin	β-actin	42	1:1500 (for salispheres homogenates) 1:4000 (for tissue homogenates)	Sigma

Table 2.1 Primary antibodies used in all chapters.

As 4e-bp1 expression ranges between hypophosphorylated to hyperphosphorylated, in these series of experiments, the hyperphosphorylated form was indicated as the active 4e-bp1 and the hypophosphorylated form was indicated as the inactive 4e-bp1.

All immunoblots were probed overnight with primary antibodies expect for two hours with β-actin. Goat anti-mouse HRP or anti-rabbit HRP antibodies were used as secondary antibodies for two hours at room temperature (Dako Ltd, Ely, UK), followed by chemiluminescent detection.

2.9 Densitometry

Band intensities of protein of interest were quantified by the Lab Image software (Bio-Rad Laboratories, Hertfordshire, UK). Acquired values were normalized to the values of β-actin.

2.10 MTS viability assay

The MTS assay was processed by adding 20 µl of the MTS reagent (Promega, Southampton, UK) to 100 µl of cells or salispheres in 96 wells plate according to the manufacturer protocol. Following 3 hours of incubation, the proliferation was determined at an absorbance of 490 nm by the Bio-Rad microplate reader (Bio-Rad, Hertfordshire, UK). To insure equal density, cells were cultured at day 0 in 96 wells and processed for MTS assay depending on the experimental design.

2.11 Organoid formation assay

To examine the ability of salispheres to form ductal-acinar like structure, salispheres were cultured in collagen/matrigel matrix according to the Nanduri et al. (2014) protocol. Briefly, salispheres were collected at day 4 of culture and pelleted at 400 *g* for 5 minutes. The pellet was resuspended at a ratio of 40 µl of collagen in DMEM/F-12. 60 µl of matrigel was added to the mixture and the total volume of 100 µl was plated into a flat-bottom 96-well plate. The collagen/matrigel matrix was then incubated at 37°C for approximately 15 minutes to allow the matrix to solidify. SC medium containing 10% of fetal calf serum (FCS) was added. Organoids were observed and imaged every two days of culture.

2.12 Tissue collection

For tissue analysis, the submandibular glands were dissected at the end of the experiments and divided into two pieces. One piece was homogenized for biochemical analysis and the second piece was fixed in 4% formalin for histological analysis.

2.13 Gland homogenization

10 mg of tissue was homogenized in a homogenizing buffer solution to for a 5% (w/v) homogenate by the MP FastPrep-24 homogenizer (MP Biomedicals, LLC, USA). The homogenizing buffer solution consists of 1% TritonX-100, 1 mM EDTA and 1% v/v dilution of protease inhibitor cocktail set 1 (Merck Chemicals Ltd, Nottingham, UK). The volume of homogenizing buffer solution used was calculated as below:

Gland weight x 19 = volume of homogenizing buffer required

The samples were centrifuged and supernatants were collected for biochemical analysis.

2.14 Protein concentration quantification

Protein concentrations of submandibular gland tissue homogenates were measured in 96-well plates using the BCA assay (Thermo Scientific, IL, USA), according to the manufacturers' instructions. A dilution of 1:100 of the homogenates was used and the results were compared to a standard curve of BSA.

2.15 Periodic Acid Schiff's staining

A Periodic Acid Schiff's staining (PAS staining) protocol was used to determine and evaluate glycoproteins. After gel electrophoresis, the gel was fixed in 25% methanol and 10% glacial acetic acid for one hour. Then, the gel was washed in water for 20 minutes followed by incubation for 15 minutes in 2% (w/v) periodic acid. The gel was washed with distilled water and incubated with Schiff's reagent in the dark for one hour until pink bands are developed.

2.16 Coomassie Brilliant Blue staining

After electrophoresis, gels could be stained with 25 ml of Coomassie Brilliant Blue R-250 solution plus 10 ml of 10% acetic acid solution for 20 minutes. The gels were then washed with 10% acetic acid only prior to imaging the gels.

2.17 Tissue sections and histochemical staining

Tissue samples were fixed overnight in 4% formalin and dehydrated in an ascending alcohol series before embedding in paraffin. Sections were cut using the microtome at a thickness of 5 µm and mounted on super-frost plus coated slides.

Slides were incubated on a heat block at 60°C for one hour followed by de-waxing, whereby the slides were incubated twice in xylene; a first incubation for 5 minutes and a second for 10 minutes. Next, the slides underwent 3 incubation steps (3 minutes each) in 100% industrial methylated spirit followed by washing under running water.

2.18 Haematoxylin and Eosin staining

Haematoxylin and Eosin staining (H&E) was used to assess the general morphology of the tissue section. The samples were stained for 3 to 5 minutes in Mayer's Haematoxylin (Thermo Fisher Scientific, Leicestershire), washed for 2 minutes in running water, differentiated in 1% acid alcohol and stained for 3 minutes in Eosin (VWR International, Leicestershire, UK). The slides were then washed in running water and dehydrated in 100% industrial methylated spirit, air–dried, cleared and mounted with DPX mounting solution (Sigma Aldrich, UK).

2.19 Morphometric assessments

The histochemical staining of submandibular glands was used for measuring 25-40 random acini (μm^2) per sample and the mean area calculated using the Image J software version 1.46 (NH Maryland, MD, USA).

2.20 Immunohistochemistry

For immunohistochemistry, tissue sections were firstly de-waxed, dehydrated in xylene, followed by incubation in ethanol and rehydrated in distilled water. Next, the sections were treated with 3% hydrogen peroxide for 10 minutes to reduce the endogenous peroxidase. Sections were further treated with preheated citric acid buffer solution (pH 6.0), as an antigen retriever. Sections were cooled in cold water and incubated with 2% BSA blocking solution to avoid unspecific binding. Later, sections were incubated with the primary antibody at room temperature to localize the protein of interest. Next, the sections were washed with 1% TBST prior to incubation with the secondary biotinylated antibody for 30 minutes (Dako, UK). Then, they were incubated for another 30 minutes with streptavidin-biotin complex

horseradish peroxidase (StreptABC-HRP; Vector Laboratories, UK). The visualization of the peroxidase activity was carried out by diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, UK). The DAB was applied to the sections for 10 minutes, followed by nuclear counterstaining for 30 seconds with Mayer's haematoxylin.

2.21 Statistical analysis

All statistical analysis was carried out by ANOVA test followed by Tukey's test or two-tailed unpaired t-test using Microsoft Excel 2011 (Microsoft, Redmond, WA) and Prism statistical program version 19 (Hampshire, UK). Data were calculated as mean with the standard error of the mean (Mean ±S.E.M) and p-values less than 0.05 were considered statistically significant.

3 THE INFLUENCE OF mTOR AND WNT ON SALISPHERE DEVELOPMENT

3.1 Introduction

Within the mTOR pathway, mTOR is upregulated by an abundance of growth factors such as IGF, cellular energy via AMP-activated protein kinase (AMPK) or amino acids via Rag mediators (Shaw, 2008). Wnt is also associated with mTOR activity and is an important signalling protein that regulates cell proliferation, stem cell maintenance and stem cell self-renewal (Nusse et al., 2008, Choo et al., 2006). The Wnt signalling mechanism of action involves the cellular release of Wnt proteins, which binds to the Frizzled (Fz) and low-density lipoprotein (LDL) receptor complex. Upon Wnt binding to the receptor, signals are transduced to different intracellular proteins, which include Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3β), Axin, Adenomatous Polyposis Coli (APC) and β-catenin. The transcriptional regulator β-catenin is usually degraded in the absence of Wnt signals. However, in the presence of Wnt, β-catenin is accumulated in the cytoplasm and subsequently in the nucleus where it binds to DNA to initiate transcription (Figure 3.1) (Logan and Nusse, 2004).

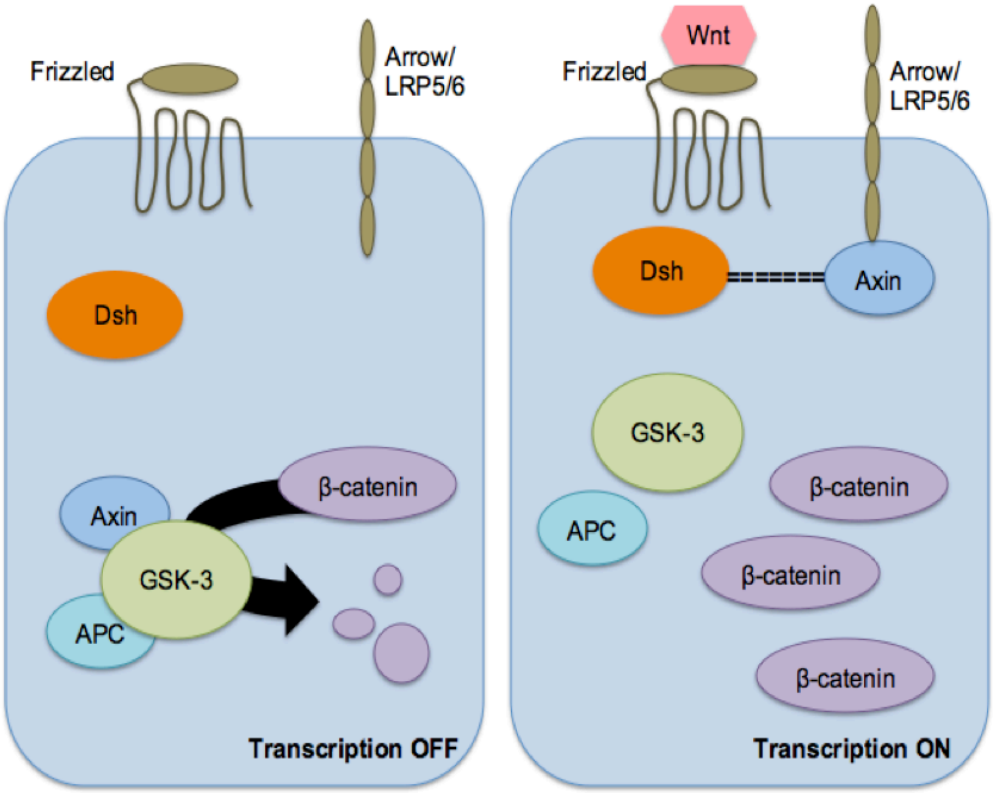


Figure 3.1 Wnt signalling in the absence and the presence of Wnt proteins. Left panel shows the degradation of β-catenin due to the absence of Wnt signals. Right panel shows the accumulation of β-catenin in the cytoplasm and in the nucleus due to the binding of Wnt protein to the Frizzled/LRP receptor, resulting in the transcriptional activation [Modified from (Logan and Nusse, 2004)].

The interaction between mTOR and Wnt might occur specifically via GSK3-β/TSC2, which negatively regulates mTOR activity (Rosner et al., 2008). Wnt phosphorylates GSK3-β, which in turns inhibits TSC2 and activates mTOR. Inoki et al. (2006) demonstrated the ability of Wnt signals in stimulating mTOR in *vitro* and *in vivo*.

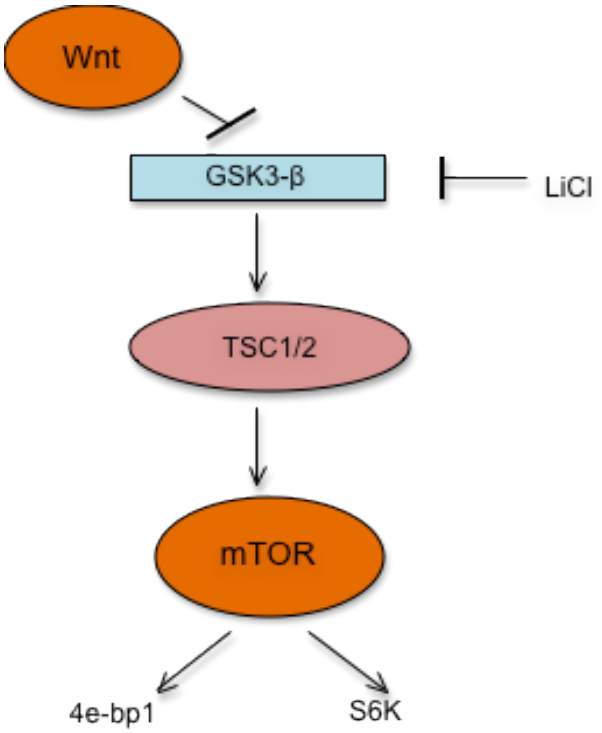


Figure 3.2 The relationship between mTOR and Wnt signaling. mTOR can be regulated by Wnt via GSK3-β, where the inhibition of GSK3-β results in the activation of mTOR via TSC2 [Modified from (Wu and Pan, 2010)].

Although, there are different classes of Wnt agonists such as Norrin and R-spondin, the GSK3-β inhibitor such as LiCl, have been also used for Wnt stimulation (Klein and Melton, 1996; Hedgepeth et al., 1997; MacDonald et al., 2009). Since GSK3-β degrades β-catenin. Interestingly, GSK3-β is a mutual component of mTOR and Wnt signalling, LiCl could stimulate both mTOR and Wnt by inhibiting GSK3-β (Figure 3.2) (Sarkar et al., 2009; Wu and Pan, 2010).

Upon activation, mTOR regulates several roles at cellular level such as protein synthesis, cell growth and cell proliferation. Protein synthesis involves three main stages: initiation, elongation and termination (Wang and Proud, 2006). The first stage of protein synthesis is regulated by 4e-bp1, whereas the other two stages are regulated by S6 kinase (S6K) (Magnuson et al., 2012).

These processes are highly regulated and occur when the main two substrates are phosphorylated. Specifically, two genes encode for SK6, *S6K1* and *S6K2*, and both are responsible for cell size and growth, whereas the 4e-bp1 regulates the cap-dependent translational machinery (Pende et al., 2000; Sonenberg and Hinnebusch, 2009; Fenton and Gout, 2011). The main substrate of S6K is S6 ribosomal protein (S6rp) (Ferrari et al., 1991). However, 4e-bp1 is classified as an inhibitor of translation initiation by binding to 4IF4E, which is a component of the 4eIF4 complex, hence regulating the cap-dependent translation process.

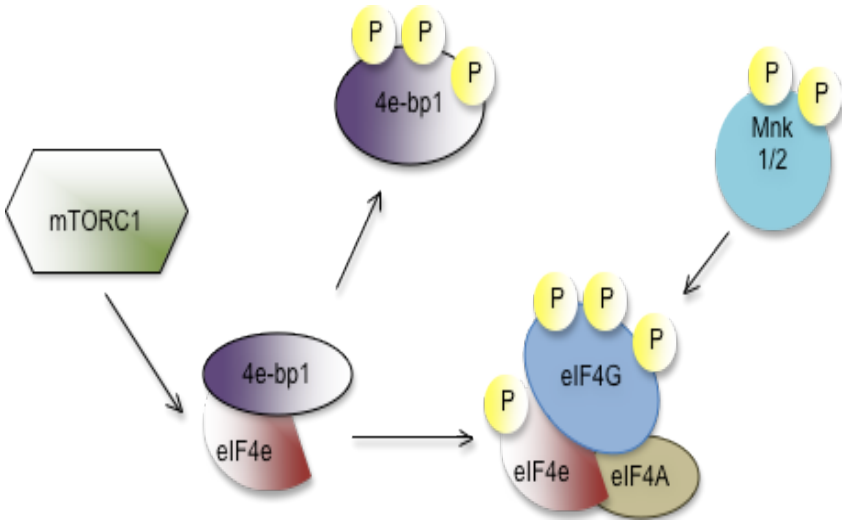


Figure 3.3 A general scheme of the translation initiation stage of protein synthesis. Inactive 4e-bp1 is usually coupled with eIF4e; however, when mTOR is activated 4e-bp1 is phosphorylated and dissociates from the eIF4e resulting in the formation of the eIF4F complex. The eIF4e and eIF4G are phosphorylated as a consequent of 4e-bp1 release, as well as a consequence of MNK1/2 phosphorylation (Hou et al., 2012).

The eIF4e complex consists of three polypeptides which are: eIF4E, eIF4A and eIF4G. eIF4E is a cap binding protein, eIF4A is a RNA helicase and eIF4G is a scaffolding protein (Sonenberg and Hinnebusch, 2009). The phosphorylation of 4e-bp1 initiates translation through the dissociation of eIF4E from 4e-bp1 and interacting with eIF4G (Heesom and Denton, 1999; Scheper and Proud, 2002). Hence, rapamycin is able to block the translation initiation through the de-phosphorylation of 4e-bp1 and the formation of the eIF4G complex (Figure 3.3) (Scheper and Proud, 2002). In addition, mitogen activated protein kinase MAPK interacting protein kinase MNK1/2 can phosphorylate eIF4E (Pyronnet, 2000). MNK1 and MNK2 were discovered by Fukunaga and Hunter (1997) and Waskiewicz and Cooper (1997). The two MNKs share a high sequence homology in their catalytic and terminal domains but MNK1 can bind both to ERK and p38 MAPK, whereas MNK2 binds to ERK only (Waskiewicz et al., 1997; Slentz-Kesler et al., 2000; Scheper and Proud, 2002).

After the initiation stage, S6K1 is recruited and phosphorylates different substrates including PDCD4, eIF4B and S6 rp. It is also important to note that S6 rp phosphorylation plays a role in cell growth (Magnuson et al., 2012). In addition, both 4e-bp1 and S6K are believed to be involved in cell proliferation and cell cycle progression. The overexpression of eIF4E is also associated with the increase in the cell size (Fingar et al., 2004). Hence, both mTOR substrates cooperate in cellular processes, albeit the role of each substrate for each process (e.g. protein synthesis) is variable.

mTOR is considered a key regulator of protein translation, therefore it is important for regulating cell growth and maintaining organ homoeostasis (Murakami et al., 2004; Lloyd, 2013). The dysregulation of mTOR is involved in aging and various diseases such as cardiac hypertrophy (Soesanto et al., 2009; Johnson et al., 2013), and tumour growth in cancer such as ovarian cancer and hepatocellular carcinoma (Zhou et al., 2010; No et al., 2011; Populo et al., 2012).

Since the mTOR network is involved protein synthesis, cell proliferation and cell growth and most importantly it is inactivated in normal salivary glands, the first objective was to understand the role of mTOR during salisphere development. In addition, the novel activation of mTOR in atrophic glands implies that it could be involved in regeneration. For these reasons, mTOR was selected as the focus of this project. Several inhibitors have been used such as rapamycin, LiCl, DKK1 inhibitor (which targets Dickkof-1) and MNK1/2 inhibitor to explore the effects of suppressing upstream and downstream regulators of mTOR on salispheres formation and development.

3.2 Materials and methods

3.2.1 Experimental design

Cultured salispheres were treated with different inhibitors at day 0 of culture. At day 4 of culture, salispheres were counted, morphologically assessed and collected for further analysis.

3.2.2 Salivary glands stem/progenitor cell culture

Salispheres were isolated and cultured as described in section 2.2. Plated cells in 12-well dishes were directly treated with the inhibitors in triplicates.

3.2.3 Treatments

Cells were treated from day 0 with rapamycin (Rapa), LiCl (Sigma, UK), a co-treatment of rapamycin and LiCl (Rapa+LiCl), mouse recombinant Dickkof-1 (DKK1) inhibitors (R&D systems, Bio-Techne Ltd, UK) and MNK1/2 inhibitors (Merck Millipore, Merck Chemicals Ltd., UK). The table below illustrates the concentrations which have been used in this chapter:

Treatment	Concentrations
Rapamycin	22 nM
LiCl	10 mM
DKK1	20 ng/ml
	60 ng/ml
	100 ng/ml
MNK1/2	2.2 μM
	3 μM

Table 3.1 Concentrations of rapamycin, LiCl, DKK1 inhibitor and MNK1/2 inhibitor used during salispheres formation (day 0 – day 4).

3.2.4 Cells/salispheres counting

Around 2 x 10^6 cell/ml of cells were plated into each well. At day 4 of culture, salispheres were counted as explained in section 2.3.

3.2.5 Imaging

Cells and salispheres were imaged at day 1, 2, 3 and day 4 of culture at a magnification of 20x as well as imaging salispheres at day 4 of culture at 10x and 40x as described in section 2.4.

3.2.6 Size of salispheres

Size of cells and salispheres was measured at day 1, 2, 3 and day 4 of culture. In addition, size of salispheres was also measured at day 4 post treatment as described in section 2.5.

3.2.7 Salispheres collection

Cells and salispheres were collected at day 0, 1, 2, 3 and day 4 of culture as explained in section 2.6.

3.2.8 Immunoblotting

Gel electrophoresis and immunoblotting were carried out to measure mTOR activity as described in section 2.7.

3.2.9 Antibodies

All immunoblots were probed overnight at 4°C in TBST containing 5% BSA including p-4e-bp1 and p-S6 rp and anti β-actin, blots were probed for 2 hours only in TBST as described in section 2.7. The table below shows the antibodies and dilutions which have been used in this chapter:

Antibody	Abbreviation	Molecular weight (kDa)	Dilution	Company
Phospho Glycogen Synthase Kinase-3	p-GSK-3β	47	1:1000	Cell signalling, New England Biolabs, UK
Phospho Eukaryotic initiation factor (4EI) 4E-binding protein (4e-bp1)	p-eIF4e	25	1:1000	Cell signalling, New England Biolabs, UK

Table 3.2 Antibodies which have been used for immunoblotting.

3.2.10 Densitometry

Quantification of band intensity was determined as mentioned in section 2.9.

3.2.11 MTS Assay

Cell proliferation levels were measured as described at day 0, 1, 2, 3 and day 4 of culture as described in section 2.10.

3.2.12 Organoid formation assay

Salispheres were cultured at day 4 in collagen/matrigel matrix according to the protocol explained in section 2.11.

3.2.13 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Tukey's test.

3.3 Results

3.3.1 Parallel relationship of mTOR to salispheres formation

The process of salispheres formation relies on the aggregation of single cells in order to form a sphere around 2 to day 3 of culture (Figure 3.4, A). The area size of salispheres showed a gradual increase over time in culture ($p<0.05$) (Figure 3.4, B). Similarly, the MTS values gradually increased, but the highest value appeared at day 3 ($p<0.02$) and day 4 ($p<0.005$) of culture compared to day 1 of culture (Figure 3.4, C).

mTOR was activated during the formation of salispheres, where the phosphorylation of mTOR substrates, 4e-bp1 and S6 rp, were clearly visible from the first day to the fourth day of culture, but unphosphorylated 4e-bp1 at day 0. The phosphorylation of S6 rp increased rapidly ($p<0.0001$) and appeared from day 0 of culture (Figure 3.5).

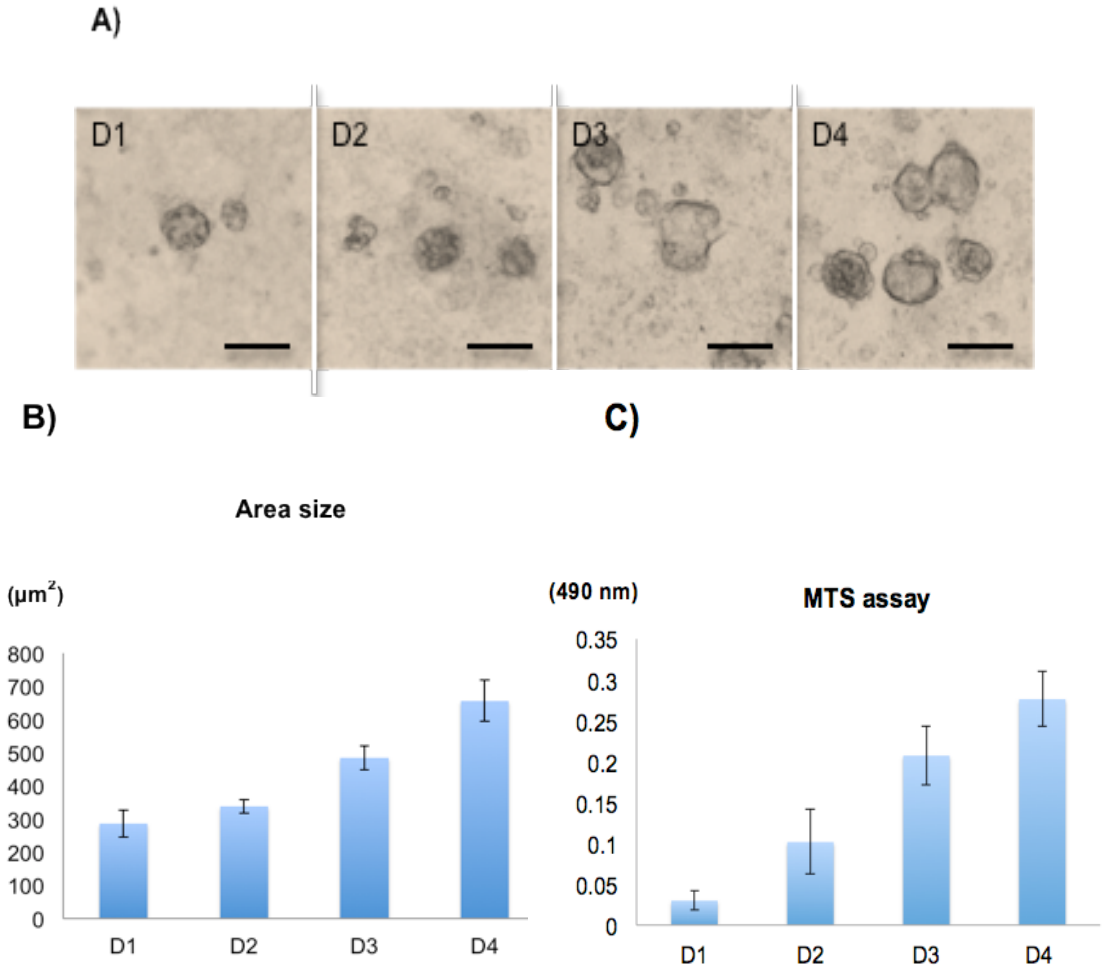


Figure 3.4 Stages of salispheres formation. A) Images showing the stages of salispheres formation (scale bar = 50 μm) B). Area size of salispheres showed a gradual increase in corresponding to the clumping of single cells to form a salisphere (*p=0.05). C) A steady increase in MTS values during the growth of salispheres in culture as the highest values appeared at day 3 (*p=0.0187) and day 4 (*p=0.0027) compared to day 1 of culture. Data represented as mean±S.E.M, n=3.

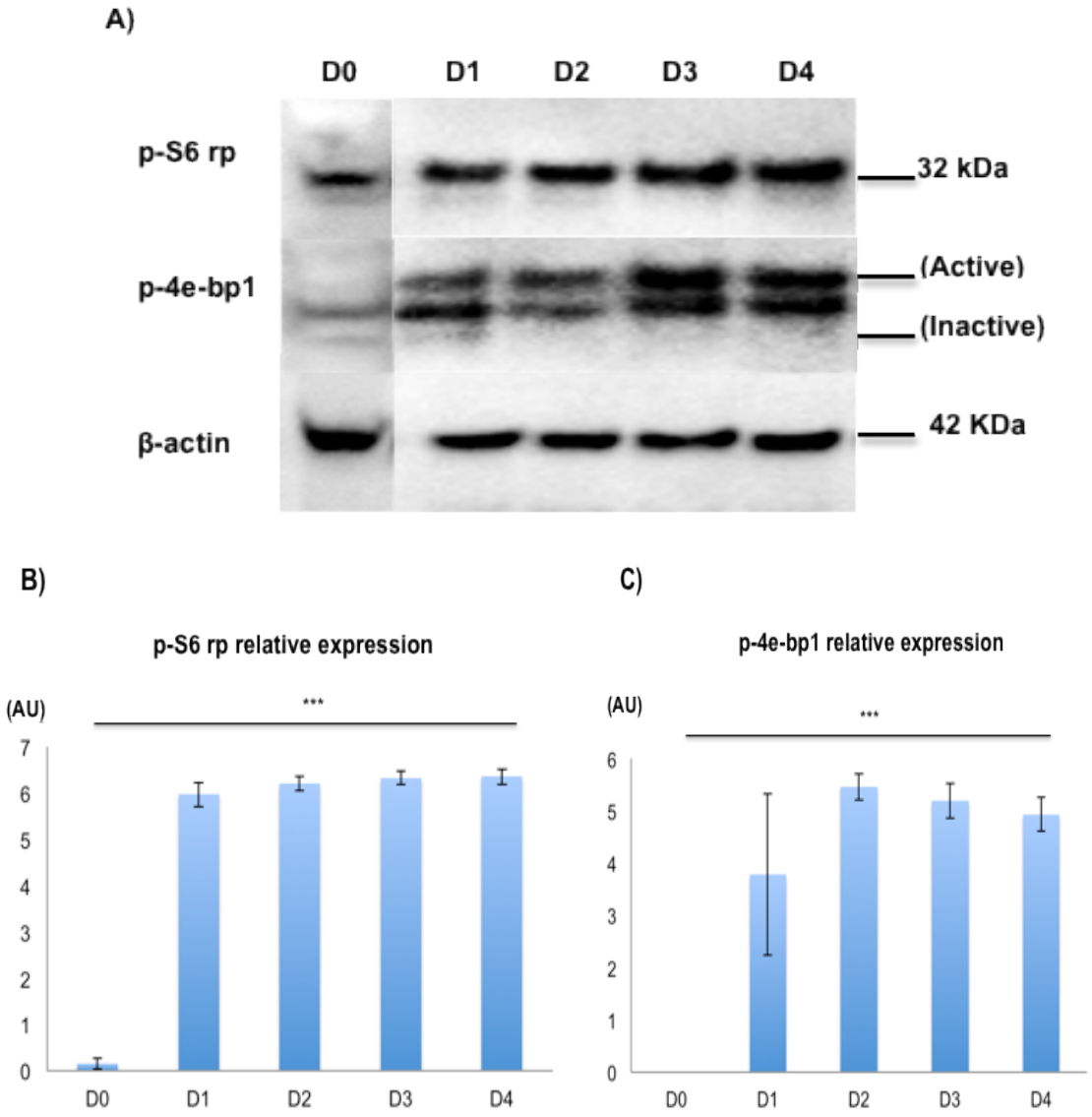


Figure 3.5 Growing salispheres *in vitro* exhibit mTOR activity. A) Immunoblot of p-S6 rp and p- 4e-bp1 showed that mTOR was active at all stages of salispheres development, except day 0 where 4e-bp1 was not expressed. B) Relative expression of S6 rp normalized to β-actin showed a gradual phosphorylation of S6 rp expect low level of expression at day 0 compared to day 1, 2, 3 and day 4 (***p<0.0001). C) Relative expression of 4e-bp1 normalized to β-actin showed hyper-phosphorylation of 4e-bp1 at all stages of salispheres formation except day 0 where 4e-bp1 was hypo-phosphorylated (***p<0.0001). Data represented as mean±S.E.M, n=4.

3.3.2 Inhibition by rapamycin demonstrates the importance of mTOR for growing salispheres *in vitro*

To understand the importance of mTOR for salispheres formation, mTOR was inhibited by rapamycin. The incubation of salispheres with rapamycin from day 0 revealed the capability of rapamycin to inhibit mTOR activity in developing salispheres as indicated by a significant reduction in size ($p<0.005$) and number ($p=0.0293$) (Figures 3.6 A, B and C). Although rapamycin significantly affected mTOR expression by complete inhibition of S6 rp ($p=0.0010$), it did not fully inhibit 4e-bp1 expression ($p=0.0029$) (Figure 3.7).

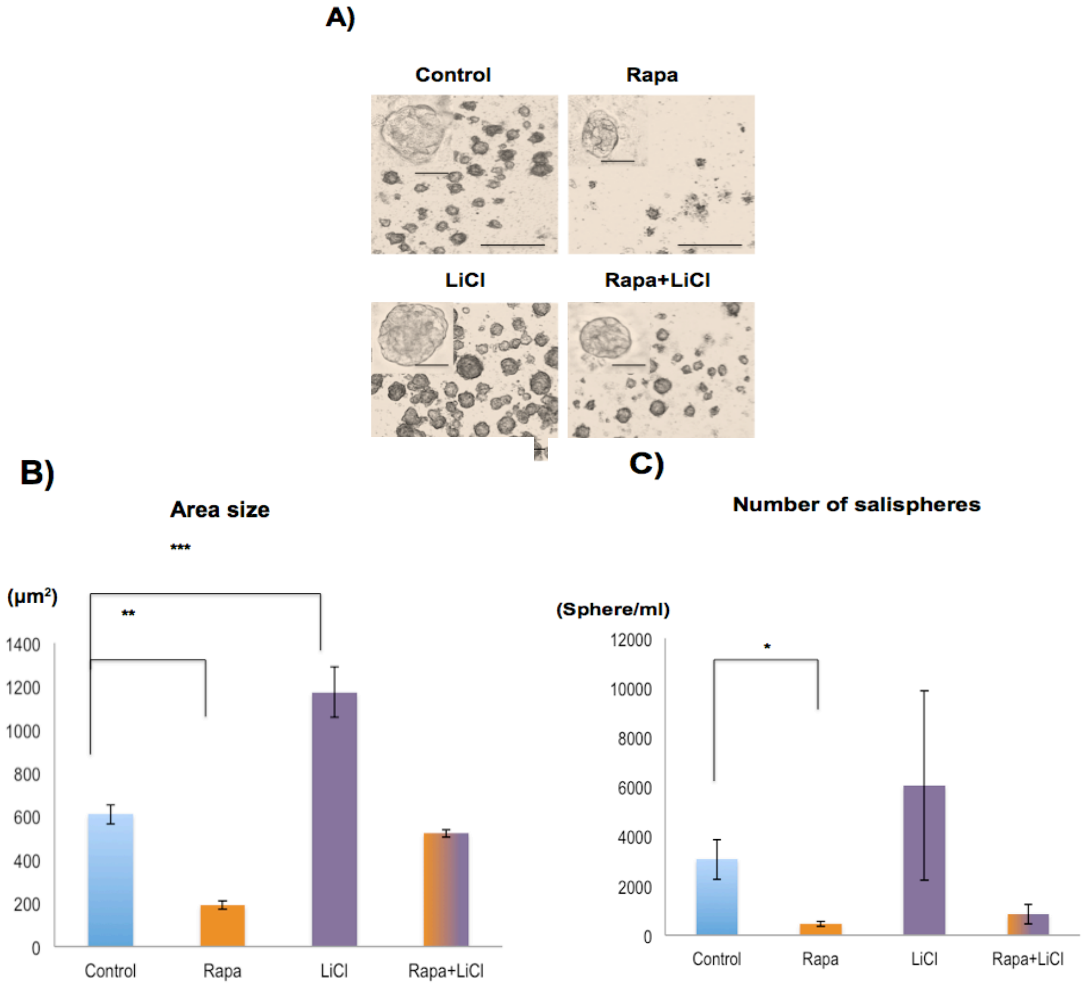


Figure 3.6 Effects of rapamycin, LiCl and the co-treatment of rapamycin and LiCl on the primary culture of salispheres. A) Images of untreated salispheres (control) and treated salispheres with rapamycin, LiCl and co-treated salispheres with rapamycin and LiCl at day 4 of culture. The small and large scales represent 20 µm and 50 µm respectively B) Size of salispheres were affected by the different treatments, specifically, rapamycin resulted in a significant reduction (*p=0.02) whereas LiCl resulted in a significant increase in size compared to the control (***p=0.0004). A slight recovery in size was observed in treated salispheres with rapamycin and LiCl (p=0.2139). C) Number of salispheres after four days of treatment was significantly affected by rapamycin. Data represented as the mean±S.E.M, n=3.

3.3.3 Modulation of mTOR activity by other mechanisms

The use of LiCl showed a significant effect on the size of salispheres (***p<0.0005) and an increase in number of salispheres, yet statistically the increase was insignificant (Figures 3.6, B and C). In terms of mTOR, LiCl might have some influence in stimulating mTOR via GSK3-β (Figure 3.8), specifically on the phosphorylation of 4e-bp1 (Figure 3.7, C), although no statistical significance was observed.

The co-treatment of rapamycin and LiCl illustrated that salispheres were not capable of overcoming the action of rapamycin. However, the presence of LiCl with rapamycin affected the recovery of size but not number of salispheres. It also showed that LiCl was not able to recover mTOR activity in the presence of rapamycin (Figure 3.7).

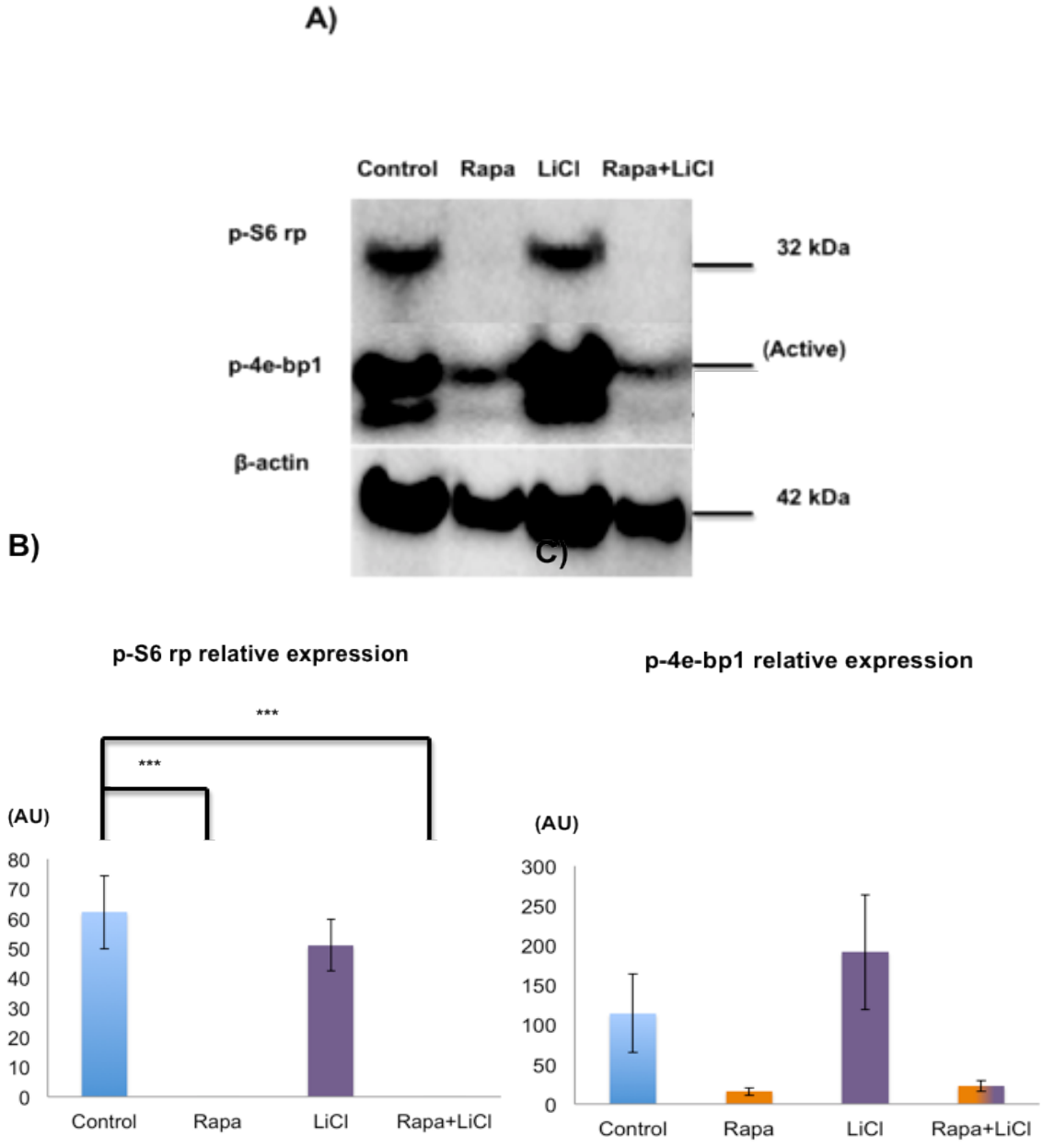


Figure 3.7 The impact of rapamycin, LiCl and the co-treatment of rapamycin and LiCl on mTOR activity. A) Immunoblots of salispheres lysates, which were probed with p-S6 rp, p-4e-bp1 and β-actin. B) Rapaymcin treatment alone or combined with LiCl caused total inhibition in p-S6 rp expression ($p<0.0001$). C) However, the relative expression of p-4e-bp1 was not fully abolished by rapamycin alone or in combine with LiCl. Data represented as mean±S.E.M, n=6.

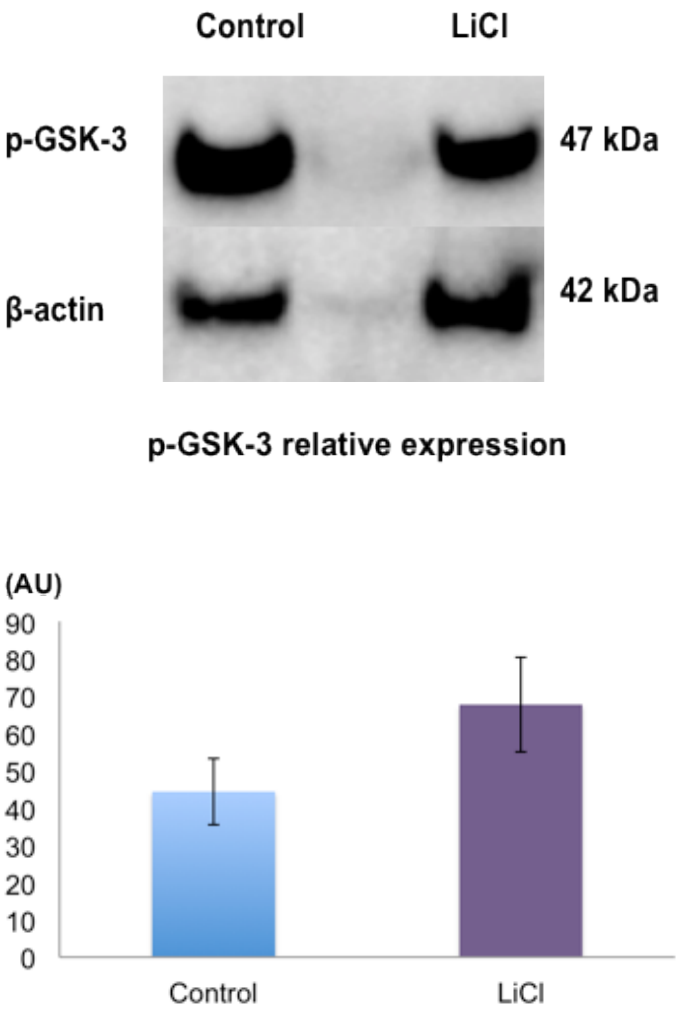


Figure 3.8 Effect of LiCl on the GSK-3 phosphorylation. A slight increase in p-GSK-3 expression in treated salispheres with LiCl, however, the increase was insignificant (p=0.2861). Data represented as mean±S.E.M, n=3.

3.3.4 Rapamycin and LiCl affected organoid formation

Untreated and treated salispheres with rapamycin and LiCl were cultured in a collagen/matrigel matrix to assess the effect of the treatments on the formation of acinar/ductal structures. Untreated salispheres were able to branch at days 5-7 of culture, while rapamycin treated salispheres did not survive. However, LiCl resulted in few formed ductal/acinar like structures compared to controls, and the majority preserved a spherical structure and increased in size overtime. Similarly, when rapamycin was combined with LiCl, salispheres preserved their spherical structure but no branching was observed (Figure 3.9).

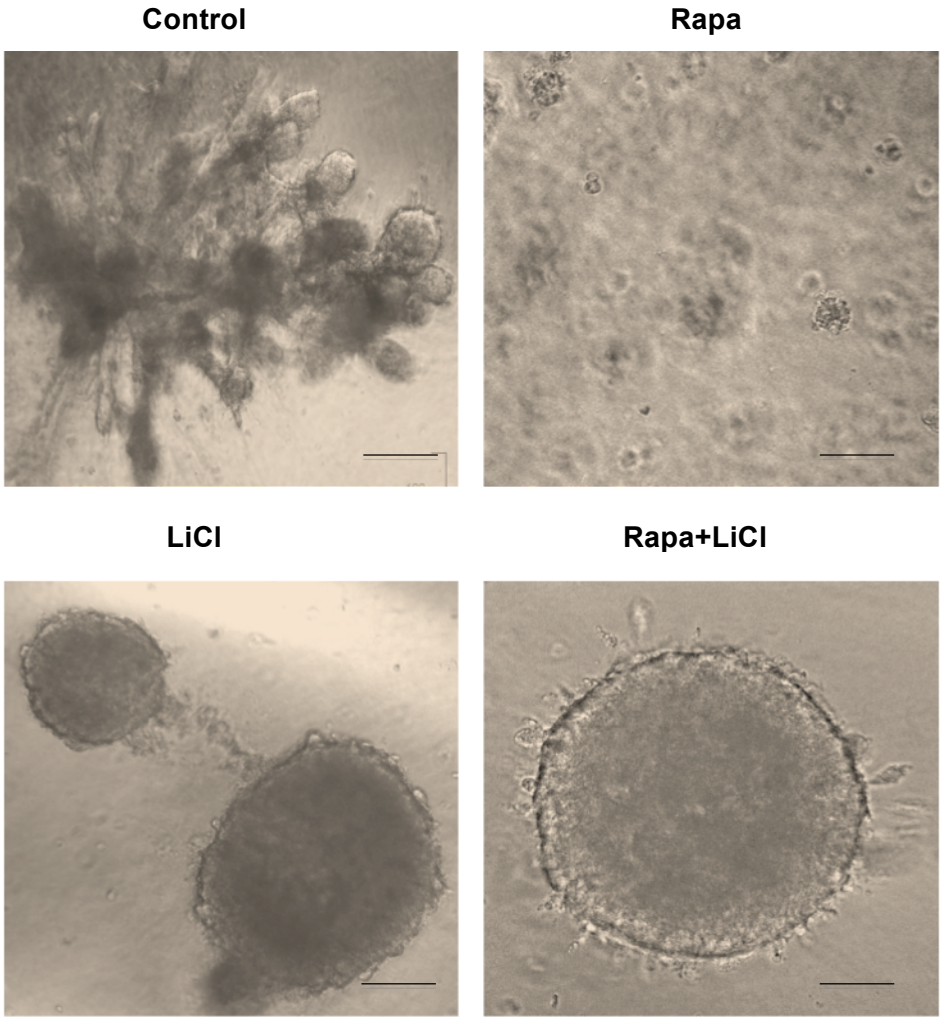


Figure 3.9 Ability of treated salispheres with rapamycin, LiCl and co-treatment of rapamycin and LiCl of forming organoids in collagen/matrigel matrix. Cultured untreated salispheres in a mixture of matrigel and collagen assisted the salispheres to develop ductal/acinar like structure. Budding of a normal sphere occurred within few days of culture, which is a similar feature to the branching morphogenesis of salivary glands. mTOR inhibition by rapamycin affected the survival of remaining salispheres. However, the administration of LiCl maintained the spherical structure of the salispheres and allowed the increase in size overtime. In addition, the use of LiCl with rapamycin occasionally allowed the survival of salispheres compared to rapamycin alone. The scale bar represents 20 μm.

3.3.5 Wnt is not the main factor for mTOR activation

Since LiCl is not a specific stimulator for mTOR as it acts on GSK-3β and it is a common component of Wnt and mTOR, a DKK1 inhibitor was introduced, which is a more specific inhibitor for Wnt signalling. Three concentrations were used to assess the optimal dose for inhibiting Wnt activity ranging from 20 to 100 ng/ml. The three doses of DKK1 inhibitor neither affected the formation nor the development of salispheres (Figure 3.10, A). Statistically, there was no difference in the number of salispheres between the control and the concentrations which were used ($p=0.1896$). However, the highest concentration of DKK1 inhibitor only showed an effect on the size of salispheres ($p= 0.0066$) (figures 3.10, B and C). In terms of the effect of DKK1 inhibitor on mTOR expression, the treatment neither reduced S6 rp expression ($p=0.2028$) nor 4e-bp1 expression ($p=0.9958$), where the expression of both mTOR substrates were comparable to the control (Figure 3.11).

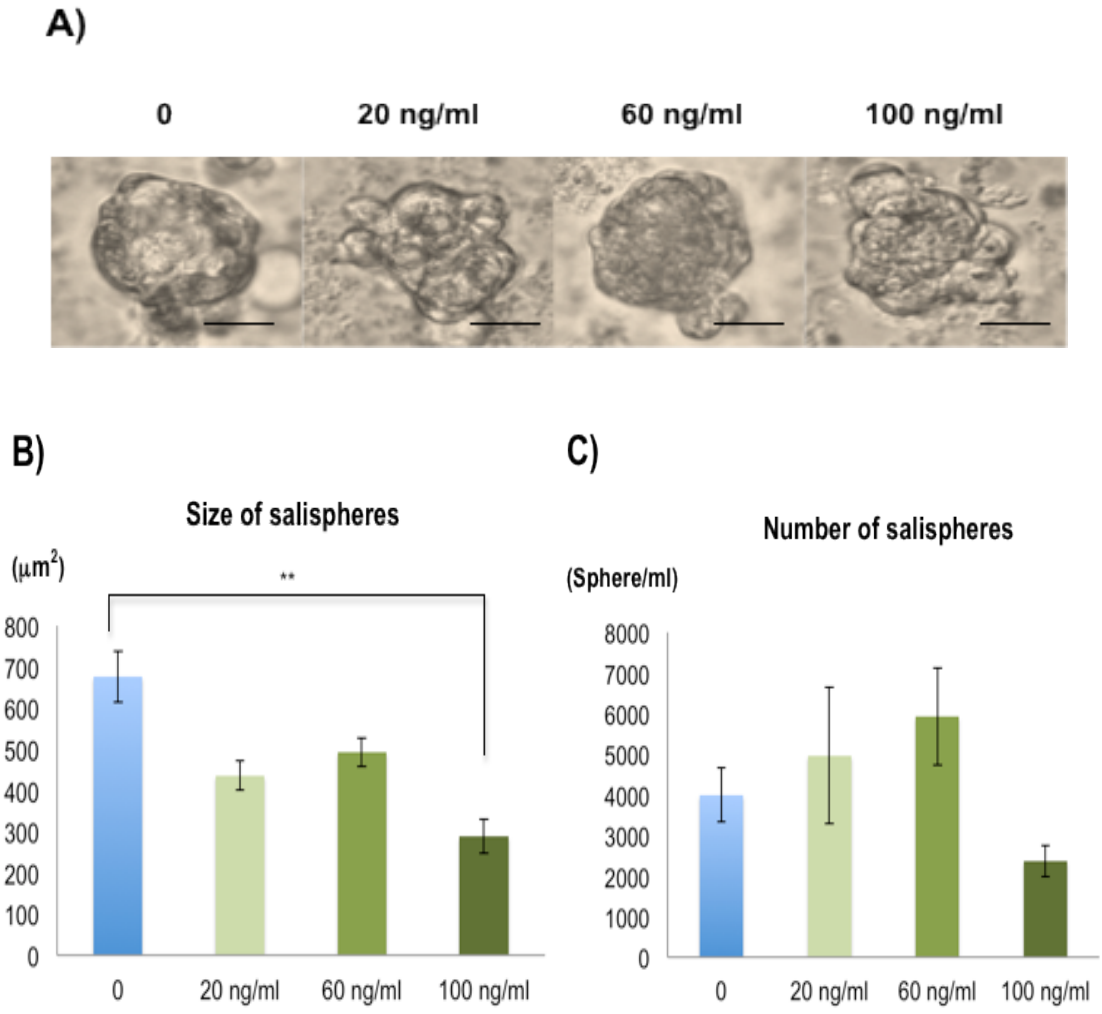


Figure 3.10 Effects of different concentrations of DKK1 inhibitor on size and number of salispheres. A) Treated salispheres with different concentrations of DKK1 inhibitor did not prevent the formation of salispheres. The scale bar represents 20 µm. B) Size of salispheres was significantly affected only when salispheres were treated with the highest concentration of DKK1 inhibitor compared to the control (*p=0.0066). C) Number of salispheres showed some variation but the differences were statistically insignificant (p=1.896). Data represented as the mean±S.E.M, n=3.

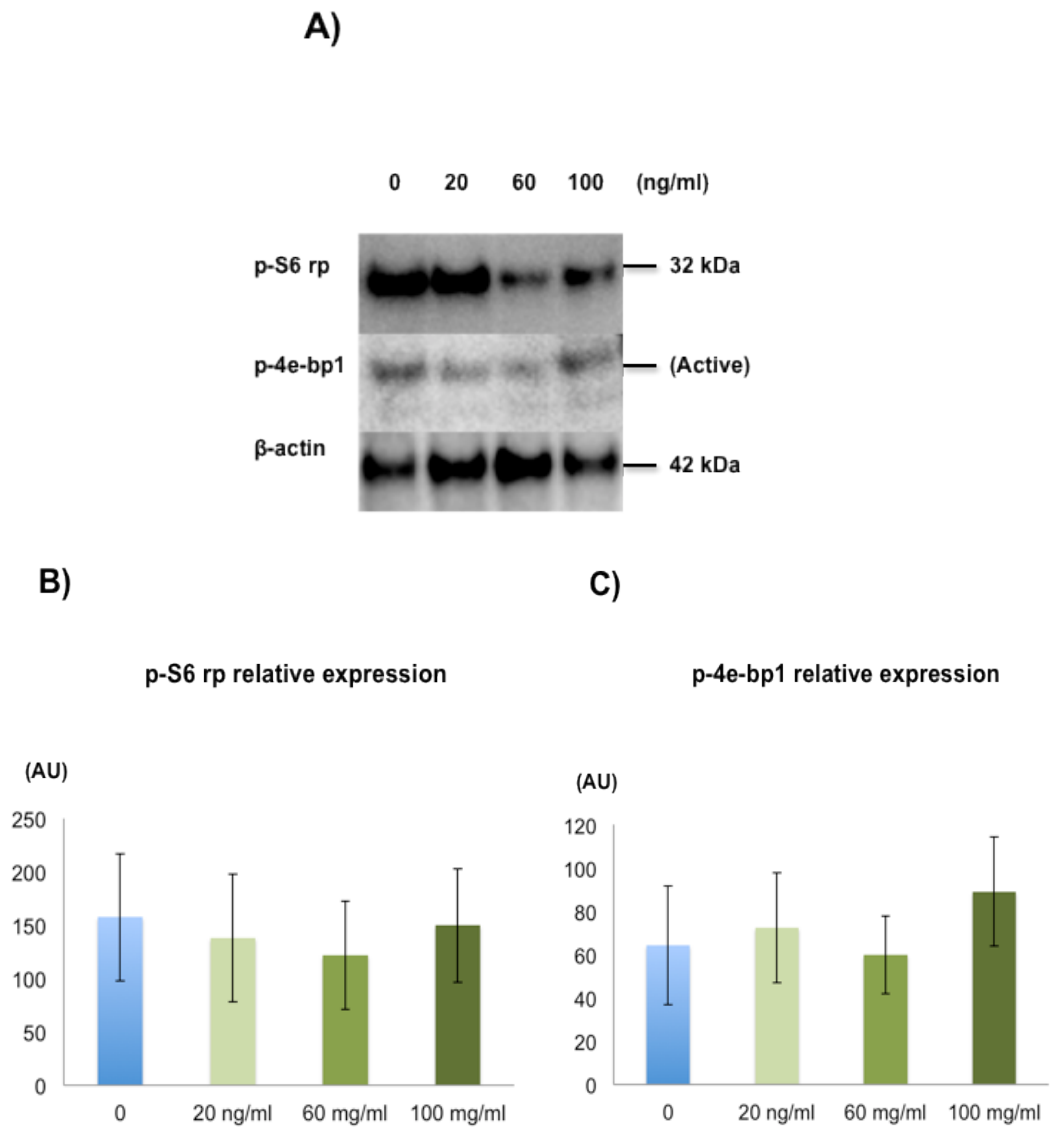


Figure 3.11 mTOR status in treated salispheres with 20, 60 and 100 ng/ml of DKK1 inhibitor. A) Immunoblots of treated salispheres with three doses of DKK1 inhibitor, which were probed with mTOR substrates antibodies. B) mTOR was not affected by DKK1 inhibitor as the phosphorylation of S6 rp was comparable to the control (p=0.9722). C) In addition, the different ranges of DKK1 inhibitor did not show any significant effects on expression of 4e-bp1 (p=0.8362). Data represented as the mean±S.E.M, n=6.

3.3.6 MNK1/2 inhibitor showed an effect on salispheres formation

The three ranges of MNK1/2 concentrations used showed variable effects on salisphere culture. The formation of salispheres was affected by the MNK1/2 inhibitor, where the size of salispheres was gradually reduced with increasing the concentration of MNK1/2 inhibitor ($p=0.0037$) (Figure 3.12). However, number of salispheres was variable among the different concentrations of MNK1/2 inhibitor and the inhibitor did not show any significant differences ($p=0.0865$). Interestingly, the MNK1/2 inhibitor showed a significant reduction in the phosphorylation of 4e-bp1 only but not s6 rp, but insignificant decrease in eif4e phosphorylation (Figure 3.13).

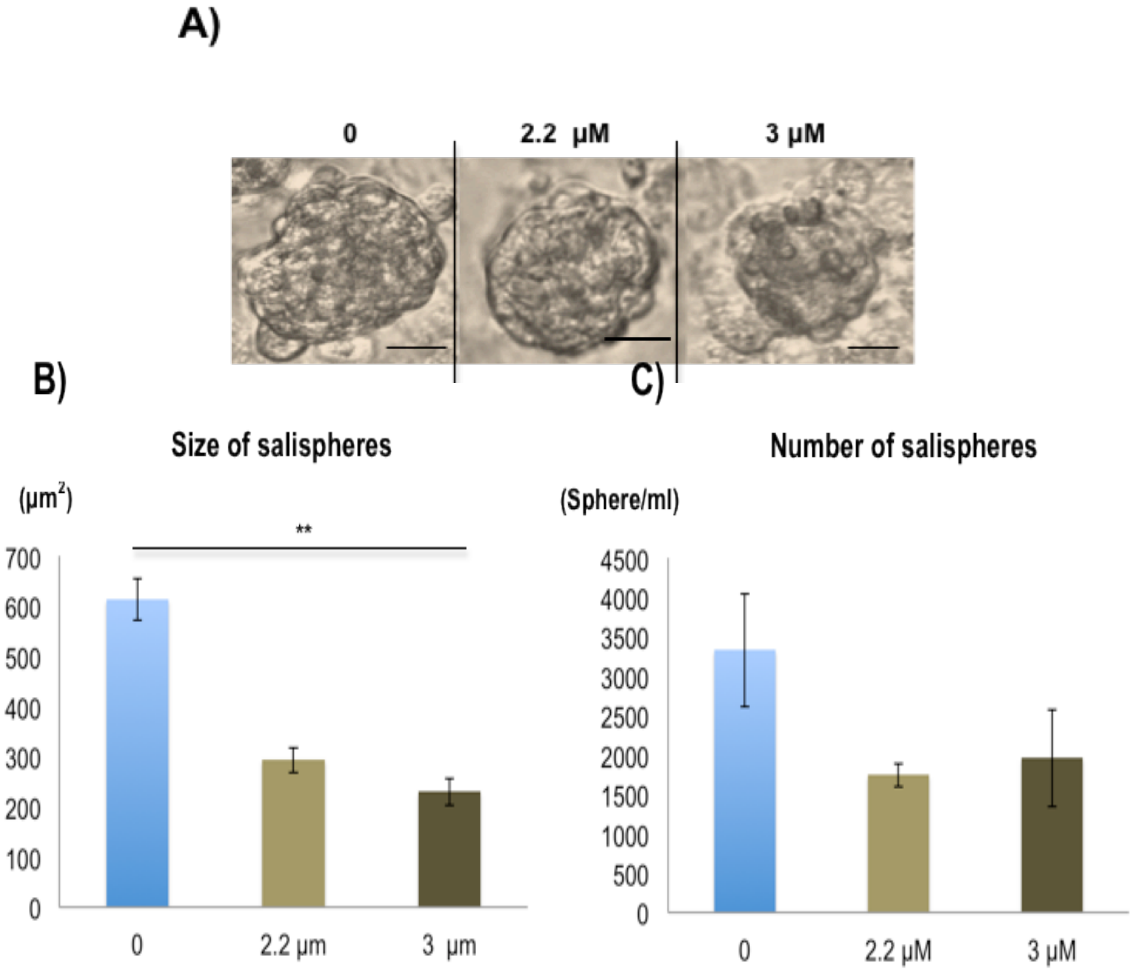


Figure 3.12 Effects of MNK1/2 on salispheres culture. A) Images of salispheres with/out 2.2 µM and 3 µM of MNK1/2 inhibitor. The scale bar represents 20 µm. B) Average size area of salispheres was reduced with an increase in the concentration of MNK1/2 inhibitor. C) Number of salispheres was not significantly affected by the treatment. Data represented as mean±S.E.M, n=3.

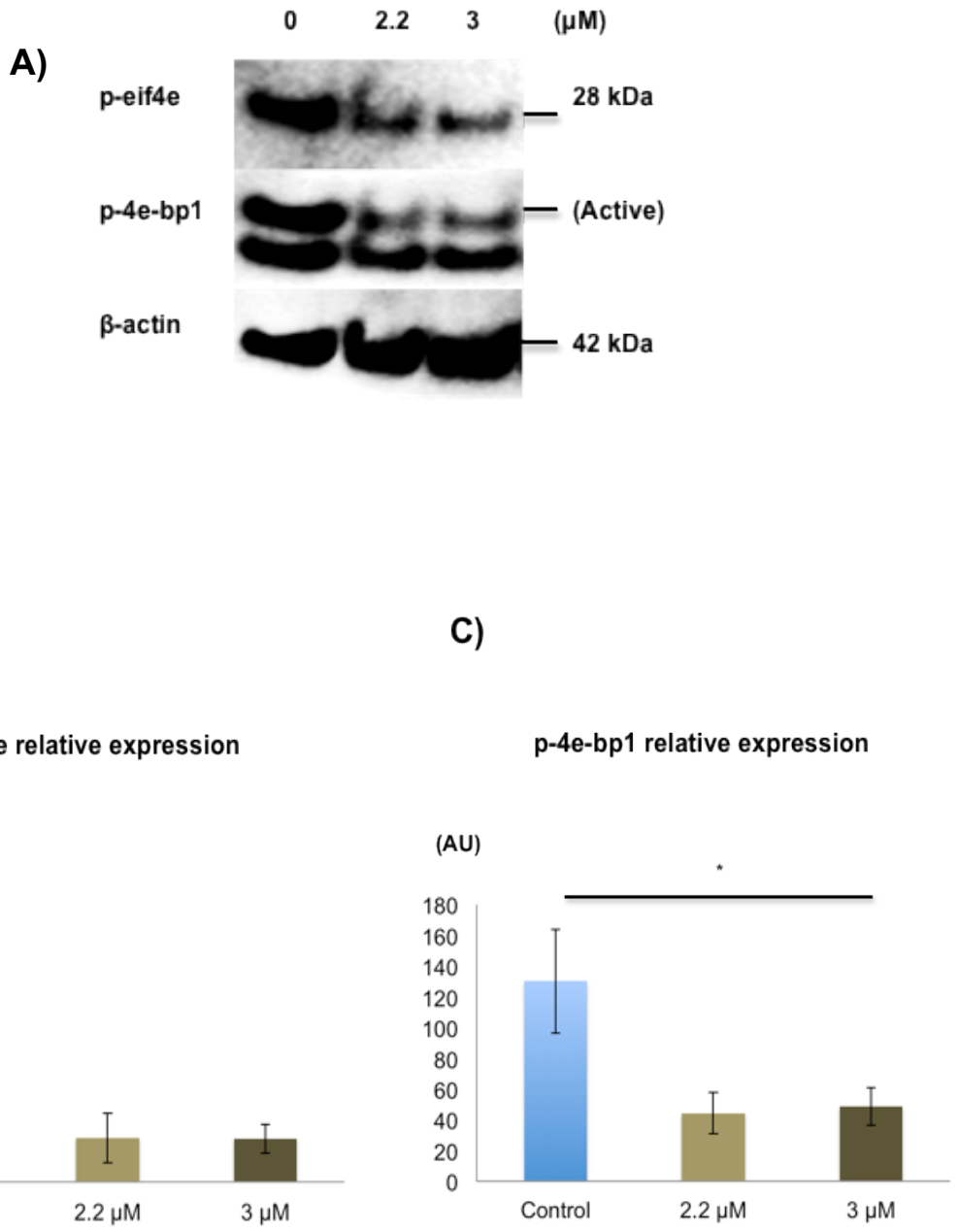


Figure 3.13 mTOR status in salispheres post treatment with 2.2 μM and 3 μM of MNK1/2 inhibitor. A) Immunoblot showing the effects of MNK inhibitor on the phosphorylation of eif4e and 4e-bp1. B) Relative expression of eif4e was slightly suppressed by the action of MNK inhibitor. C) A gradual decrease in the phosphorylation of 4ebp1 paralleled increasing the concentration of MNK1/2 inhibitor. However, only 4e-bp1 expression was significantly affected by the MNK1/2 inhibitor ($p=0.0382$). Data represented as mean±S.E.M, n=4.

3.4 Discussion

Although mTOR activity drives cap-dependent protein translation, it is inactivated in normal mature salivary glands. Activity can be induced by the ductal ligation of the submandibular gland (Silver et al., 2010; Bozorgi et al., 2014) and therefore thought to be important for regeneration, and potentially for stem/progenitor cells. As previously shown, mTOR is associated during salivary gland atrophy; this study was designed initially to understand if mTOR is important for growing salispheres *in vitro*.

Unlike normal salivary glands, cultured salispheres *in vitro* exhibit mTOR activity, with a gradual increase during the formation and development of salispheres. Presumably, tissue digestion and culture is sufficient to activate mTOR by day 1, as they require insulin for their culture and insulin is one of the upstream regulators of mTOR in several different cell types (Scott et al., 1998; Wang et al., 2006).

The parallel increase in size, number and mTOR activity during the formation of salispheres implies that mTOR is a factor involved in the formation of salispheres, as it is considered master of cell growth (Laplante and Sabatini, 2009). This observation is supported by mTOR inhibition by rapamycin, which illustrated how crucial mTOR is for salisphere growth (Fingar et al., 2002; Inoki et al., 2006). Similar to work on salispheres, Paliouras et al. (2012) has shown that neurosphere formation decreased with increased concentrations of rapamycin, and mTOR also plays a role in maintaining neural progenitor pools (Sato et al., 2010; Paliouras et al., 2012).
There are several upstream regulators of mTOR activity such as growth factors and Wnt signalling. For this reason, LiCl was introduced in this study as it functions through the GSK-3, resulting in the activation of mTOR.

The action of LiCl on enhancing mTOR via GSK-3 and TSC2 interaction has shown several impacts on salispheres growth. Interestingly, LiCl resulted in a significant increase in the size of the salispheres and slight increase in their numbers (Figure 3.6). It also showed that it has some influence on mTOR activity, specifically the expression of 4e-bp1, but not the expression of S6 rp (figure 3.7). The observation that LiCl can activate both Wnt signalling and mTOR via GSK-3, brought into question which signalling pathway (mTOR or Wnt) was responsible for the increase of size and numbers of salispheres (Silva et al., 2010; Phiel and Klein, 2001). Although the phosphorylation of GSK-3 was slightly increased in the LiCl treated salispheres (Figure 3.8), this may indicate that LiCl influenced mTOR activity.

The dual treatment of rapamycin and LiCl suggested that LiCl may act solely via the mTOR pathway rather than activating a separate pathway such as Wnt (Montagne et al., 1999). LiCl treatment suggests that Wnt signals were not able to recover mTOR activity in the presence of rapamycin. This may highlight GSK-3 as a regulator of mTOR activity as it has been demonstrated that deleting GSK-3 affected neurosphere proliferation (Ka et al., 2014). In addition, the co-treatment of rapamycin and LiCl illustrated that mTOR is an essential factor of salisphere growth; hence, the combination would not assist the maintenance of salispheres by inhibiting mTOR and stimulating Wnt as observed in hematopoietic stem cells (Huang et al., 2012). This implies that mTOR activity is crucial for the survival of salispheres.

As LiCl could stimulate both mTOR and Wnt signalling, the DKK1 inhibitor was introduced, which acts by binding to LRP6 in order to prevent Wnt and frizzled interaction (Niehrs, 2006). The effect of the DKK1 inhibitor would explain if Wnt is an essential upstream regulator of mTOR. The effects of the DKK1 inhibitor and

rapamycin treatments on salispheres suggest that mTOR is a fundamental pathway for their development (Maimets et al., 2016). The DKK1 inhibitor significantly affected the size of salispheres but not mTOR signalling, suggesting that other upstream regulators might be responsible for keeping mTOR active in the absence of Wnt signals which is the usual stimulator of stem cells (Figure 3.11) (Nusse, 2008). Although the highest dose of the DKK1 inhibitor showed some effects on the culture of salispheres, the effects caused by rapamycin was still more potent than the effects caused by the DKK1 inhibitor. This might suggest that the potency of the inhibitor varies among each other as IWR-1 for example has shown considerable effect on salispheres culture compared to IWP-2 (Maimets et al., 2016).

In contrast to the upstream regulators, evaluating the effect of inhibiting the downstream effectors of mTOR by MNK1/2 inhibition, which is involved in the assembly of the 4efie complex, helped in understanding different aspects of mTOR inhibition (Proud, 2015). The low level of p-eif4e expression in treated salispheres suggests that the translational process is reduced but still active, which may improve their survival (Figure 3.12). However, this reduction was not potent as the effects caused by rapamycin. As previous study found that both MNK1/2 are responsible for the phosphorylation of ei4fe, but not responsible for cell growth and development (Ueda et al., 2004). This also suggests that 4e-bp1 expression might be the factor for keeping the translational machinery switched on in the absence of MNK1/2 action. In addition, it has been shown that the de-phosphorylation of eif4e is dependent on 4e-bp1 levels and MNK1/2 is not essential for the cap-translational process (Knauf et al., 2001; Muller et al., 2013).

In conclusion, examining different aspects of mTOR illustrated the necessity of mTOR signaling for healthy growing salispheres. The use of rapamycin emphasized the importance of mTOR for developing salispheres and the potency of rapamycin as a mTOR inhibitor. Furthermore, the effects of LiCl on salisphere culture suggest that mTOR and Wnt stimulation are involved in salisphere branching.

4 SALISPHERE DEVELOPMENT FROM NORMAL, LIGATED and DE-LIGATED GLANDS

4.1 Introduction

In humans, there are several conditions that can contribute to the atrophy of salivary gland such as sialadenitis, Sjögren's syndrome and radiotherapy. Ligation of the main excretory duct in rodents is commonly used to understand salivary atrophy as the histological appearance is similar to human disease (Cummins et al., 1994; Silver et al., 2010).

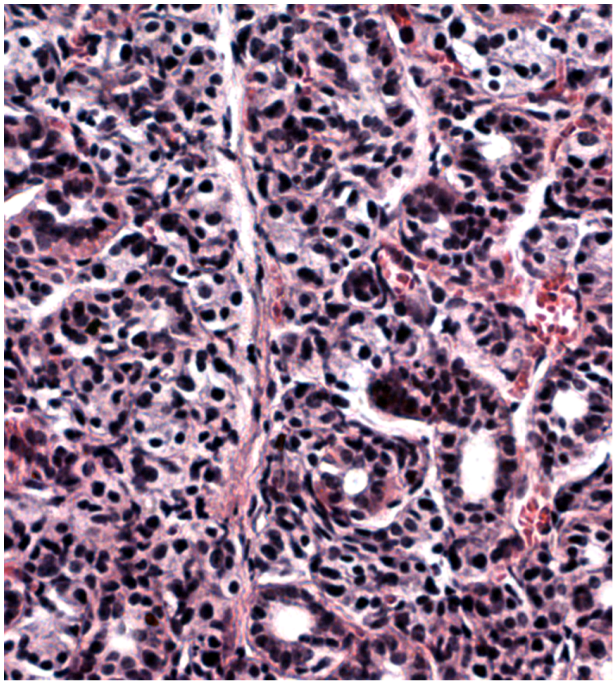


Figure 4.1 Histology of submandibular gland after seven days of ligation (with courtesy of Naomasa Kawashima).

Introducing injury through ligation of the main excretory duct causes the salivary glands to atrophy. The atrophy of the gland includes apoptosis, deletion of acinar cells, necrosis, autophagy and extensive proliferation of the myoepithelial cells, as well as mitotic proliferation of ductal cells (Burford-Mason et al., 1993; Burgess et al., 1996). The removal of acinar cells by apoptosis in chronic sialadenitis and Sjögren's syndrome is comparable to experimental ligation-induced atrophy, which makes this

experimental procedure a useful model for understanding salivary glands atrophy (Kong et al., 1997; Harrison and Badir, 1998; Takahashi et al., 2000).

Removal of the ductal obstruction leads to the regeneration of salivary glands showing an improvement in the glandular weight, branching structures and the area size of the acini (Cotroneo et al., 2008). The ligation/de-ligation procedure also induces the expression of the neonatal proteins such as SMG-B1 and B2, which are normally expressed in the acinar cells of developing salivary glands and the intercalated duct of adult salivary glands. Another protein named parotid secretory protein (PSP) is only expressed during the development of salivary glands, but no expression observed in adult mature salivary glands. An injury model results in the expression of the embryonic prenatal pathways in the acinar cells, emphasizing the regenerative capacity of salivary glands post-injury (Cotroneo et al., 2010).

The de-ligation of the salivary gland duct helps in understanding the mechanism by which salivary glands can regenerate post-injury. Earlier studies believed that regeneration could occur from resident acinar cells, whereby they proliferate directly after the removal of injury and from remaining ductal cells (Bhaskar et al., 1966; Tamarin, 1971a; Tamarin, 1971b; Takahashi et al., 2004). In the context of salivary gland regeneration, label-retaining studies have provided evidence of the presence of stem/progenitor cells in the parenchymal compartments, similar to the histological studies in regenerating salivary glands. The presence of a stem/progenitor cell pool is important for tissue maintenance, lineage commitments and differentiation to different salivary gland cell types (Lombaert et al., 2011; Chibly et al., 2014). Several studies have shown the location of these progenitor cells in the ductal compartment

and their ability to producing both acinar and ductal cells (Denny and Denny, 1999; Man et al., 2001; Nanduri et al., 2011).

As previously stated, mTOR is inactive in adult rodent normal salivary glands, but 4e-bp1 is hypo-phosphorylated and is localized mainly in acinar cells. However, mTOR becomes activated during ductal ligation and the expression of 4e-bp1 is localized around the lobular structure, suggesting that mTOR is playing a role in protecting remaining acinar cells (Silver et al., 2010). In addition, the inhibition of mTOR may also delay atrophy following ductal ligation of the submandibular gland, specifically atrophic acinar cells (Bozorgi et al., 2014).

In this chapter, mTORC2 will be briefly explained as ductal ligation, which has been carried out in this chapter, affected salisphere culture. Also the second complex of mTOR may play a role in migration and cytoskeletal rearrangement. Although less is known about the function of mTORC2 compared to mTORC1, (mainly due to the lack of a specific inhibitor for mTORC2 activity), mTORC2 is thought to regulate several substrates such as AKT, SGK1 and PKCα (Laplante and Sabatini, 2009).

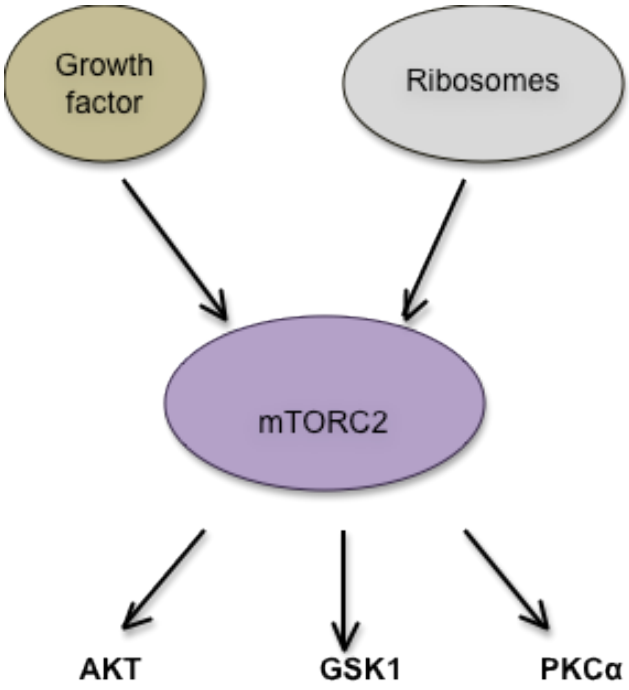


Figure 4.2 Upstream and downstream effectors of mTORC2. The activation of mTORC2 by growth factors and ribosomes is thought to affect AKT, GSK1 and PKCα. mTORC2 activation is important for cell survival and cytoskeletal rearrangement (Laplante and Sabatini, 2009; Zinzalla et al., 2011).

Growth factors are not only responsible for activating mTORC1, but also mTORC2 via the PI3K. However, the activation beyond the PI3K is distinctive between the two complexes (Zinzalla et al., 2011; Sparks and Guertin, 2010).

Unlike mTORC1, mTORC2 is considered insensitive to rapamycin, but prolonged treatment with rapamycin in certain cell types can inhibit mTORC2 (Sarbassov et al., 2006; Sparks and Guertin, 2010). The second generation of mTOR inhibitors such as torin1 has been designed to target both complexes mTORC1 and mTORC2 (Guertin and Sabatini, 2009).

Several studies have shown the involvement of mTORC2 in cytoskeletal rearrangement. For example, mTORC2 has shown a role in neuron morphology,

suggesting that mTORC2 aberration can be related to brain diseases (Angliker and Ruegg, 2013). In addition, the role of mTORC2 in the cytoskeletal remodeling can also affect the lineage selection of mesenchymal stem cells (Sen et al., 2014). However, the second complex of mTOR is not the only factor that acts on cytoskeletal rearrangement, there are other signalling pathways, which are associated with the cytoskeletal remodeling such as RhoA/ROCK signalling.

Rho kinase is a serine/threonine kinase discovered to be an effector of the small GTPase Rho (Leung et al., 1995, Amano et al., 2010). Rho kinase is comprised of two members ROCK1 and ROCK2. Both ROCK1 and ROCK2 share 62% of their overall amino acid identity and have similar substrate specificity. They are expressed in most tissues. However, ROCK2 appears to be highly expressed in brain and muscles, whereas ROCK1 is highly expressed in non-neural tissues (Leung et al., 1996; Nakagawa et al., 1996). It is responsible of the organization of the cytoskeleton, formation of stress fibers and focal adhesions, as well as cell polarity (Amano et al., 2010). The activation of ROCK by the RhoA consequently phosphorylates myosin light chain (MLC) and LIMK, which in turns inactivate cofilin (Maekawa et al., 1999; Riento and Ridley, 2003b).

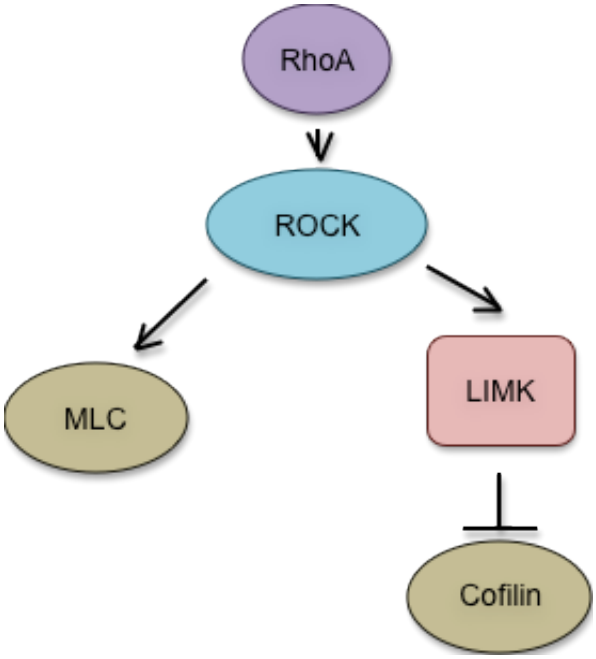


Figure 4.3 RhoA/ROCK signaling. ROCK activation relies on RhoA activity. The activation of ROCK by RhoA leads to the phosphorylation of MLC and the phosphorylation of LIMK, which inhibits cofilin. This cascade results in actin cytoskeletal rearrangement (Riento and Ridley, 2003b).

ROCK can be inhibited by the antagonist, Y-27632 (referred as ROCKi in this study) (Ishizaki et al., 2000). ROCK inhibition has shown a role in axonal regeneration in injured central nervous system (Fournier et al., 2003). In addition, it has also shown a role in the survival of cryopreserved human embryonic stem cells (Li et al., 2009, Martin-Ibanez et al., 2009).

The objective of this chapter was to determine the effect of injury on salisphere culture and to investigate if mTOR activity is affected in grown salispheres from injured glands.

4.2 Materials and methods

4.2.1 Experimental design

Salispheres were isolated and cultured from ligated and de-ligated glands for 4 and 8 days. Similarly, normal salispheres were cultured with ROCKi (10 μM) for 4 days and 8 days. All salispheres were morphologically assessed at day 4 of culture and collected at day 8 of culture to evaluate the morphological similarities and the expression levels of cytoskeletal proteins.

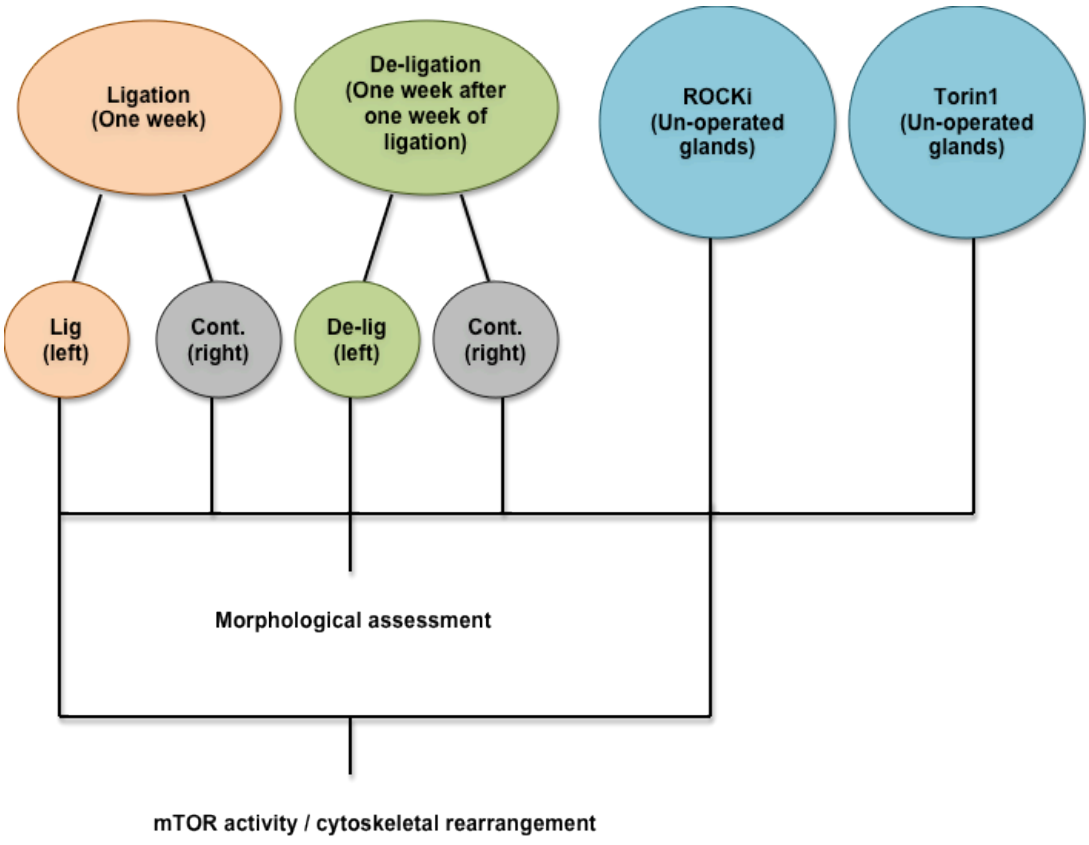


Figure 4.4 A schematic demonstrating the experiments performed in this chapter. Salispheres were cultured from ligated/de-ligated glands and collected at two-time points; day 4 and day 8. Next, only salispheres from ligated glands were used to compare the morphological similarities and differences to torin1 and ROCKi treated salispheres, to also assess mTOR activity and expression levels of cytoskeletal proteins.

4.2.2 Ligation/de-ligation of submandibular glands

Ligation was performed firstly by anesthetizing the mice with xylazine (5 mg/Kg) /ketamine (25 mg/Kg) i.p injections. After reaching sufficient depth of anaesthesia, a 0.5 cm skin incision was made in the centre of the neck. A blunt dissection was performed, where the fat surrounding salivary glands was dispersed and the left submandibular excretory duct was ligated by a 6-0 Ethivon suture (Johnson and Johnson Intl, Brussels, Belgium) or a metal clip. Following the surgery, the neck was sutured and the mice were allowed to recover from anesthesia. The mice were administered analgesics (buprenorphine, 10µg/kg) and were maintained in a warm room. Seven days post ligation; the mice were terminally anaesthetized with an overdose of pentobarbitone. For de-ligation experiments, mice were anesthetized as previously after seven days of ligation with a xylazine/ketamine i.p injection and the clip was removed. The neck was sutured and the mice were allowed to recover as previously explained. The mice were terminally anaesthetized after seven days of de-ligation and given an overdose of pentobarbitone. At the end of the experiment, the submandibular glands were collected and weighed before processing for salisphere culture.

4.2.3 Salivary gland stem/progenitor cell isolation and culture

Salispheres were isolated and cultured according to the protocol described in section 2.2. Gland weights were taken into consideration for determining volumes required of collagenase, hyaluronidase and $CaCl_2$ in the digestion step. In addition, cells were plated according to the gland weights to maintain similar seeding densities, as ligation and de-ligation were performed in one gland/animal.

4.2.4 Torin1 and ROCKi treatments

Cultured salispheres from un-operated glands were treated at day 0 with 10 nM of Torin1 (R&D systems, UK) and 10 μM of ROCK inhibitor (Millipore, Hertfordshire, UK). The selection of treatment concentrations was determined by preliminary experiments.

4.2.5 Salispheres counting

Salispheres from ligated, de-ligated glands and the contralateral glands were processed for counting at day 4 of culture by determining the average of 3 fields at 10x magnification.

4.2.6 Size of salispheres

The size of salispheres from ligated and de-ligated glands was measured using Image J software at day 4 of culture, as described previously in section 2.5.

4.2.7 Salispheres imaging and collection

Salispheres were imaged prior to collection at day 4 and day 8 of culture. ROCKi treated salispheres were imaged at day 4, 6 and 8 of culture as previously described in section 2.4 and collected as described in section 2.6.

Adherent cells were collected at day 8 of culture from ligated and ROCKi treated samples, including the un-operated floating salispheres. Adherent cells were collected after washing with PBS and scraping with RIPA lysis buffer.

4.2.8 Immunoblotting

Gel electrophoresis and immunoblotting were carried out as previously described in section 2.7.

4.2.9 Antibodies

Antibodies	Abbreviation	Molecular weight (kDa)	Dilution	Company
Phospho-focal adhesion kinase	p-FAK	70	1:1000	Cell Signalling, UK
Cytokeratin 5/8	CK5/8	54/52	1:1000	BD Pharmingen, UK

Table 4.1 Antibodies used in this chapter.

All immunoblots were probed overnight including p-4e-bp1 and p-S6 rp as previously described in section 2.8.

4.2.10 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Turkey's multi-comparison test as previously described in section 2.21.

4.3 Results

4.3.1 Glandular assessment

Ligation of the main duct of the submandibular gland resulted in a significant reduction in the weight of submandibular glands ($p=0.0002$) (40.8±2.6g, n=5) compared to un-operated glands (62.9±1.2g, n=5), whereas the contralateral gland was significantly larger than the control ($p=0.0006$) (82.2±4.6g, n=5). The removal of the clip affected the glandular weights, where a slight recovery was observed compared to a ligated gland (49.3±3.8g, n=5) ($p=0.0232$) (Figure 4.5).

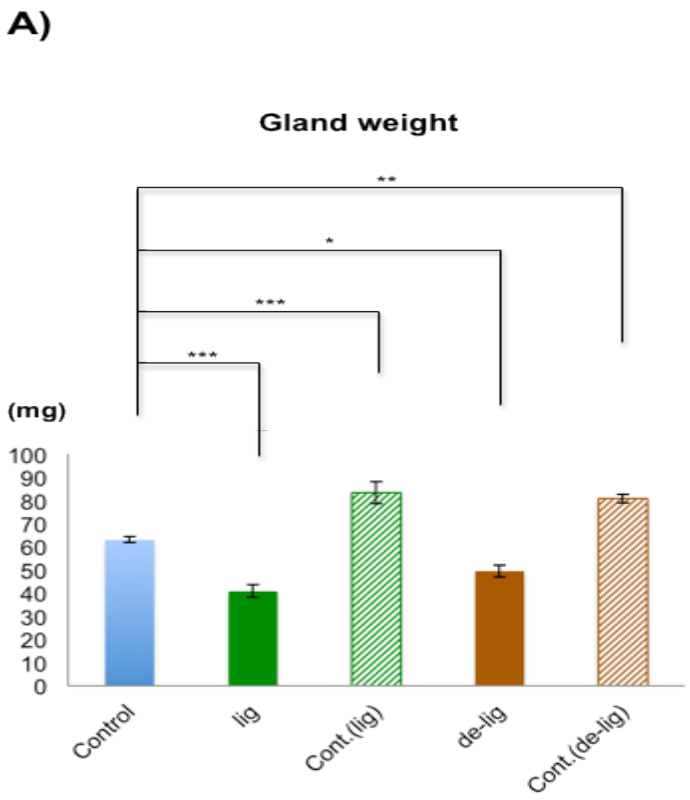
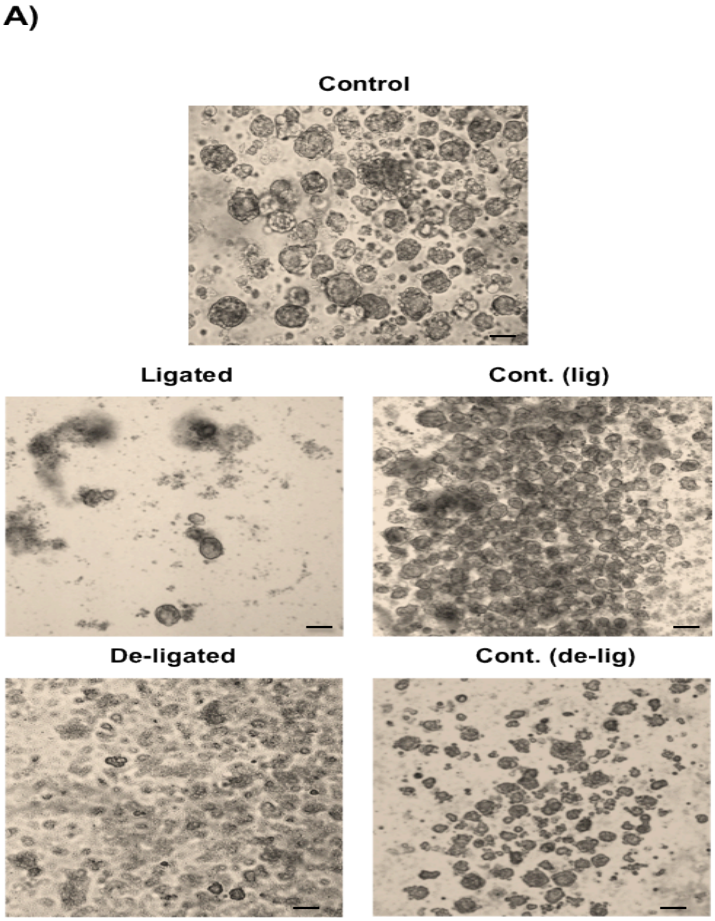


Figure 4.5 Effect of ligation/de-ligation on gland weights. Ligation of the submandibular glands (lig) caused a significant reduction in gland weights (p=0.0002) and a significant increase in the gland weights of the contralateral glands (Cont.lig) (p=0.0006). In contrast, de-ligation resulted in recovery of the gland weights (de-lig) (p=0.0232), whereas the contralateral glands of de-ligated glands (Cont.lig) were still significantly higher than the control (p=0.0021). Data represented as mean±SEM, n=5.

4.3.2 Injury affected salispheres culture

Injury initially affected the number and size of cultured salispheres. For example, ligation caused a reduction in number of salispheres; although, statistically the reduction was insignificant. While, the number salispheres from the contralateral gland of the ligated submandibular gland were significantly higher ($p=0.0430$) compared to the number of salispheres at day 4 of culture from un-operated glands (Figure 4.6, B). The removal of injury helped in the slight recovery of the number of salispheres, but the recovery is still very low compared to cultured salispheres from un-operated glands (Figure 4.6, C).



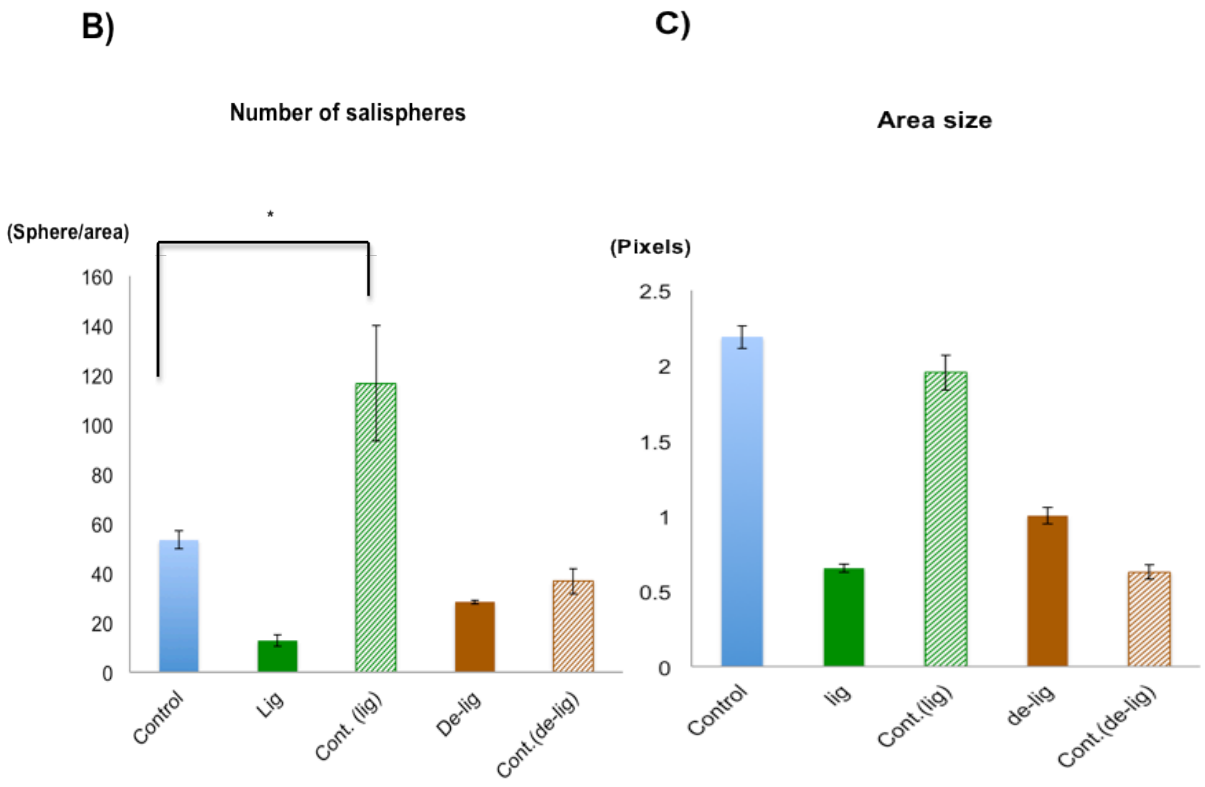


Figure 4.6 Number and size of salispheres post ligation and post de-ligation. A) Five images representing salispheres at day 4 of culture by the phase-contrast microscopy (Scale bar = 50μm). B) Number of salispheres was reduced post injury compared to the un-operated glands (control), except the contralateral gland of ligated gland showed a significant increase in number of salispheres (*p=0.0430). C) Size of salispheres were significantly reduced post injury compared to the control (***<0.0001), except cultured salispheres from contralateral glands of ligated glands (p=0.2602) was similar to the size of the control. Data represented as mean±S.E.M, n=3.

4.3.3 Injury causes morphological changes to cultured salispheres

One of the main characteristics of salisphere culture is growing in a non-adherent form. However, the salispheres post-injury behaved differently around day 6 of culture. They firstly grew in suspension (Figure 4.6, A), before adhering to the plastic dish around day 6. Later, they lost their spherical structure and spread out over time forming fibroblastic-like structures (Figure 4.8).

To assess possible reasons for these changes, two inhibitors have been used to treat salispheres from un-operated glands. Torin1 and ROCKi are inhibitors of mTORC2 and ROCK signalling respectively as both signaling pathways are involved in the cytoskeletal rearrangement.

The use of torin1 to inhibit mTORC2 initially affected the survival of salispheres, and likely inhibited both mTOR complexes. Similarly, to rapamycin, torin1 resulted in a reduction in the number of floating salispheres (Figure 4.7). In addition, no adhesion of remaining salispheres was observed in the presence of torin1. However, ROCKi affected normal salispheres similarly to salispheres derived from an injured gland. In addition, the cell viability increased over time, where the highest proliferation level was observed at day 8 of culture ($p=0.0002$) (Figure 4.9).

The expression of focal adhesion kinase (FAK) and cytokeratin5/8 (CK5) helped in determining the similarities between cultured salispheres from an injured gland and treated normal salispheres with ROCKi as ROCK is involved in the cytoskeletal rearrangement. The expression of p-FAK and CK5 was significantly higher in both cultured salispheres from ligated glands and treated salispheres with ROCKi, while CK8 expression was unaffected by ligation or ROCK inhibition (Figure 4.10).

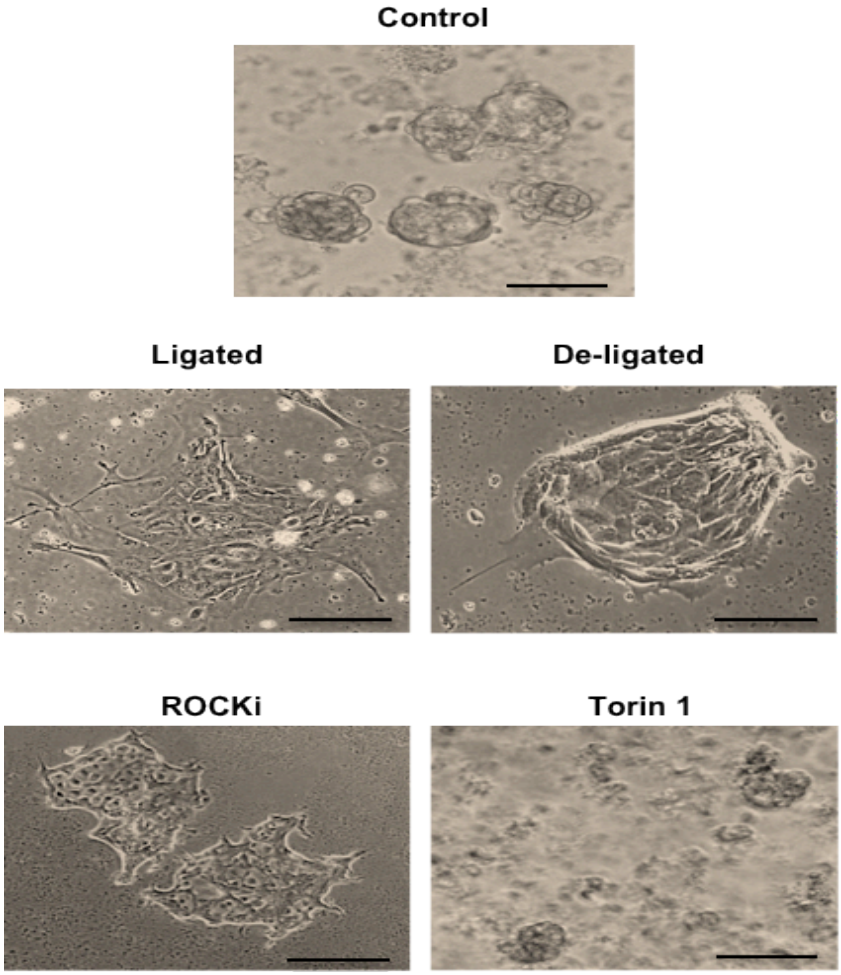


Figure 4.7 Morphological differences between cultured salispheres from un-operated glands, ligated glands, ROCKi and torin1 treated salispheres. Isolation and culture of salispheres from an injured gland resulted in the adherence of salispheres and loss of spherical structure, similar to the effect of inhibiting ROCK signaling by ROCKi. Torin1 neither helped in the survival of the salispheres nor caused them acquire similar morphology to injury. The scale bar represents 40 μm.

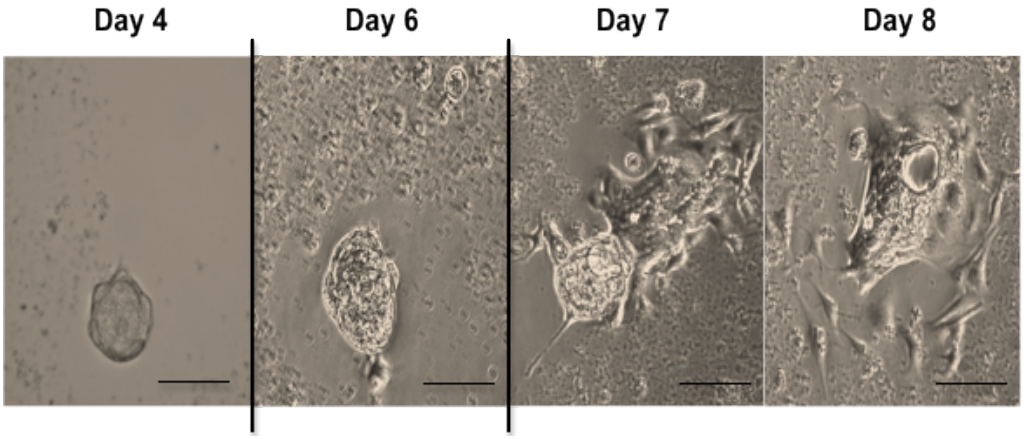


Figure 4.8 Stages of salisphere adhesion by ROCKi. Treatment at day 0 with ROCKi caused to the salispheres to adhere at around day 6 of culture. They spread out over time and the spherical structure disappeared, and was replaced with a fibroblastic-like structure after they are attached to the plastic dish. The scale bar represents 20 µm.

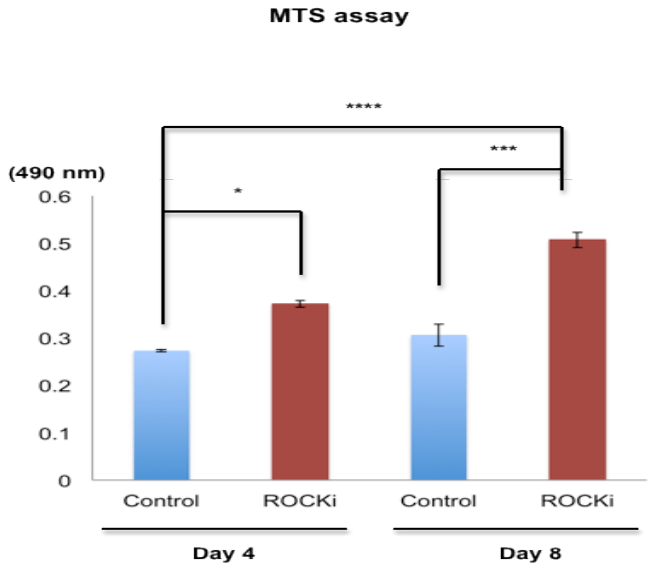


Figure 4.9 Viability with and without ROCKI at two time points. ROCKi resulted in a significant increase cell proliferation at day 4 and day 8 of culture. The treatment significantly increased the proliferation at day 4 ($p=0.0192$) and day 8 of culture (***$p<0.0001$), compared to untreated salispheres at day 4. Cell proliferation was also higher at day 8 compared to untreated salispheres at day 8 of culture (***$p=0.0002$). Data represented as mean±SEM, n=3.

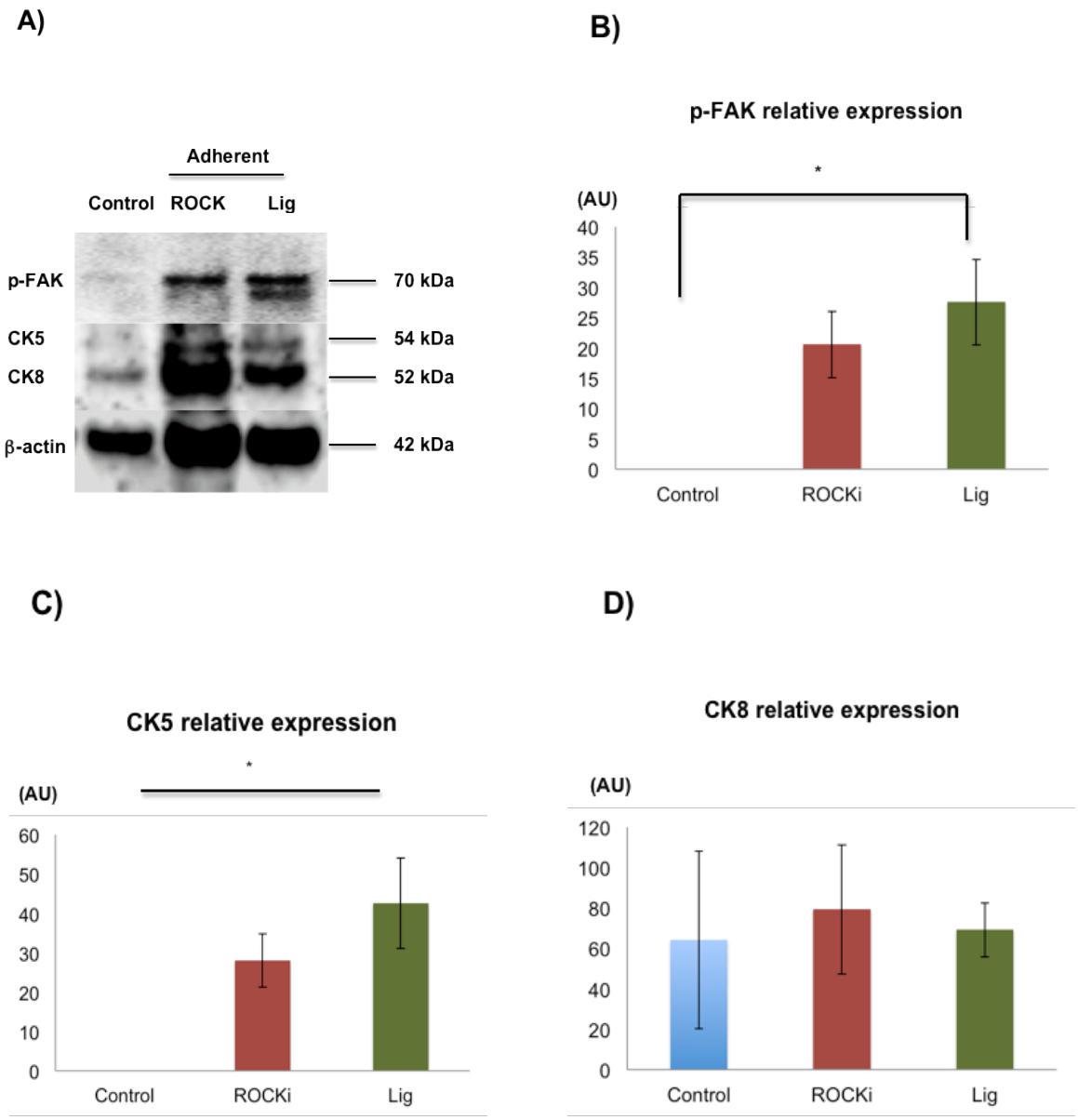


Figure 4.10 Expression patterns of p-FAK and CK5/8 in adherent salispheres in ROCKi treated salispheres and grown salispheres from ligated glands. A) Immunoblot of untreated salispheres (control), ROCKi treated salispheres and cultured salispheres from ligated glands. B) The relative expression of p-FAK was not significant in ROCKi treated salispheres ($p=0.0678$) but was statistically significant in cultured salispheres from ligated glands ($p=0.0214$). B) Both ROCKi ($p=0.0146$) and injury ($p=0.0206$) affected the expression of CK5. D) While CK8 appeared unaffected by the by ROCKi or ligation. Data represented as mean±S.E.M, n=3.

4.3.4 mTOR activity is important for protein synthesis

mTOR activity appeared to be an essential factor during normal salisphere formation and development. In this study, neither ROCKi nor ligation significantly affected the phosphorylation of 4e-bp1 and the phosphorylation of S6 rp. Although, there were some variations in the phosphorylation of S6 rp and 4e-bp1 compared to controls, the differences were statistically insignificant (Figure 4.11).

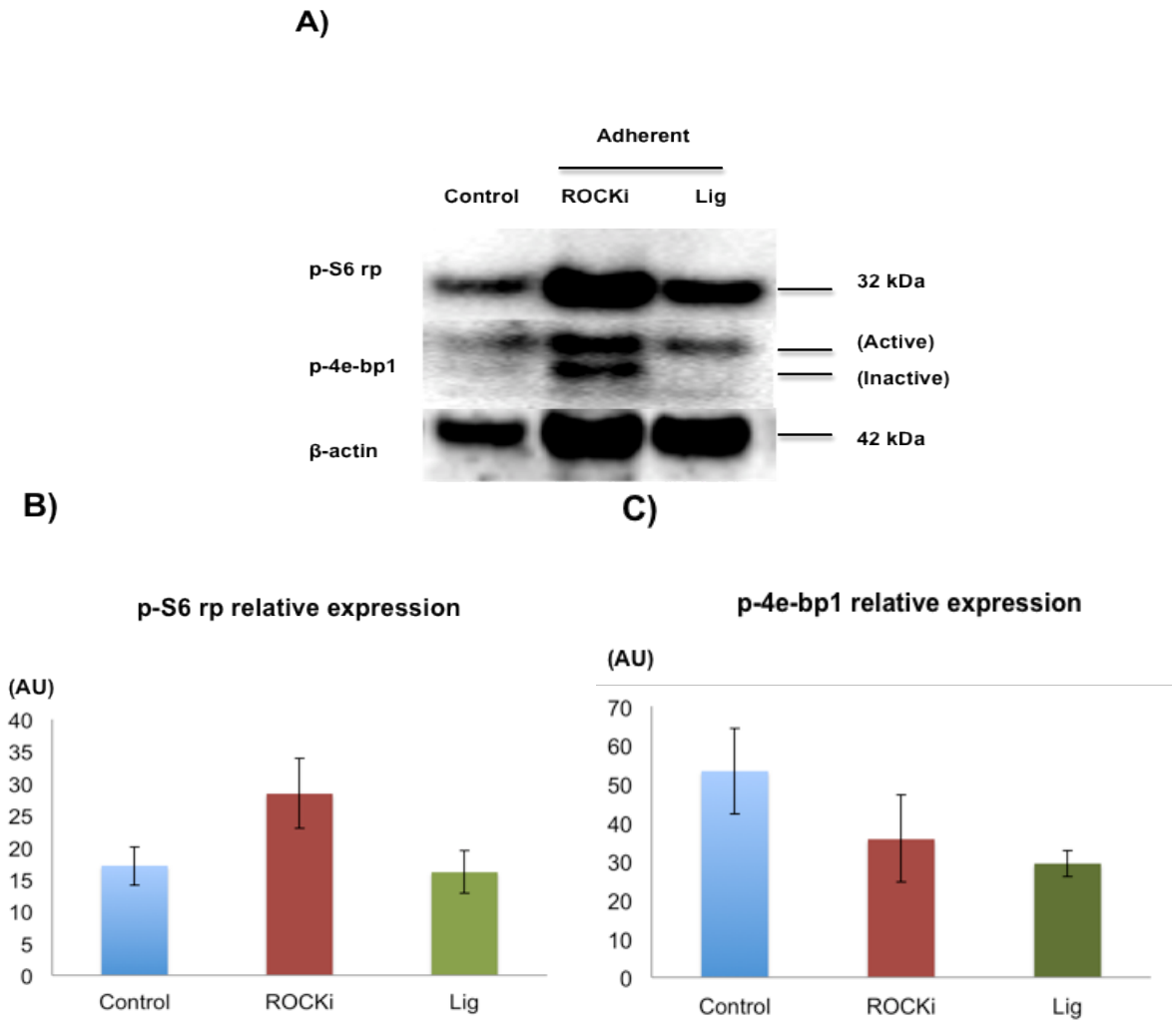


Figure 4.11 mTOR status in treated salispheres with ROCKi and in growing salispheres from ligated glands. A) Immunoblot of mTOR expression in treated salispheres with ROCKi and growing salispheres from ligated glands. B) The relative expression of p-S6 rp shows insignificant differences among the three samples. C) The relative expression of 4e-bp1 also showed insignificant differences. Data represented as the mean±S.E.M, n=3.

4.4 Discussion

Since ligation/de-ligation is considered a good model for studying regeneration, these experiments were designed to explore if salisphere formation would be affected by injury. Cultured salispheres from injured glands would help in understanding the correlation between salisphere behavior and regeneration as they are thought to represent the pool of stem/progenitor cells, which possess regenerative properties.

Grown salispheres from ligated glands illustrated that injury affected the characteristics of salispheres. First, the number of salispheres and glandular weight were significantly affected as both glandular weight and number of salispheres have been reduced, suggesting a correlation between the population of salispheres and atrophy (Bozorgi et al., 2014).

Observations from de-ligation experiments imply that salispheres are responsible for gland recovery as numbers of salispheres improved compared to those grown from ligated glands. In addition, the ability of these salispheres to repopulate seven days post de-ligation suggests that the stem/progenitor cell pool was activated.

Conversely, the significant increase in the number of cultured salispheres from contralateral glands suggests a potential compensatory hyperplasia caused by ligation. This compensatory hyperplasia was decreased after removal of the injury of the contralateral gland as the number of salispheres was reduced compared to number of salispheres during ligation. This suggests that salispheres are correlated with both atrophy and hyperplasia.

Cultured salispheres from injured glands acquired distinctive characteristics compared to cultured salispheres from un-operated glands or contralateral glands. The adhesion of salispheres following glandular injury suggests intrinsic signalling alteration occurred which affected the growth of salispheres. To investigate the possible factors that are involved in the adhesion of salispheres, mTORC2 and RhoA/ROCK were studied because both are associated with cytoskeletal rearrangements (Amano et al., 2010). Although torin1 can inhibit both complexes of mTOR, the objective of using torin1 was to explore if mTORC2 inhibition would lead to the adhesion of remaining salispheres. However, torin1 primarily appeared to affect the survival of salispheres similar to rapamycin, suggesting that mTOR signalling is fundamental to salisphere development. The effect of torin1 also suggests that mTORC2 is not associated with morphological changes and other factors that may be responsible for these alterations.

In contrast, inhibiting RhoA/ROCK caused cultured salispheres to develop similar characteristics to injured glands, suggesting that RhoA/ROCK is involved in salivary gland atrophy. This was surprising as inhibition of ROCK is widely used in stem cell culture to prevent contact-inhibition of growth and several studies have used ROCKi for cryopreservation of embryonic stem cells (Watanabe et al., 2007; Li et al., 2009).

Moreover, Lee and colleagues (2015) demonstrated that in the presence of 10 μM of ROCKi salispheres did not adhere to the plastic dish as observed in this study but prevented their senescence. Similar to results of this study, previous work has shown that ROCK inhibition effects salisphere proliferation and survival (Yu et al., 2012; Okumura et al., 2009).

The significant up-regulation of p-FAK and CK5 suggest that RhoA/ROCK may play a role in morphological alterations. Presumably, other RhoA/ROCK related markers such as MLC are also upregulated (Riento and Ridley, 2003a). Another possibility is that injury might contribute to the differentiation of progenitor cells due to cellular distress.

Since mTOR was active in cultured salispheres, experiments explored whether there are any differences in mTOR expression between growing salispheres from injured and normal glands. The presence of mTOR in all samples and the insignificance differences in S6 rp and 4e-bp1 expression imply that mTOR was not correlated to the adhesion of salispheres, but was mainly involved in cell growth. This is unsurprising because mTOR is responsible for protein synthesis (Wang and Proud, 2006). These data also suggest that the variability of mTOR expression might have occurred depending on the level of protein synthesis and cell growth, and that the molecular mechanism of mTOR is different in glands compared to cultured salispheres.

In summary, the effects caused by ligation/de-ligation to salispheres suggest that there is an association between the population of salispheres and salivary gland atrophy. The morphological similarities of salispheres between ROCKi and ligation experiments suggests that the Rho/A/ROCK might be one factor playing a role during atrophy, but also suggests that it is a complex mechanism and other factors might be responsible for morphological alterations.

5 INFLUENCE OF NEURAL INPUT ON SALISPHERE DEVELOPMENT

5.1 Introduction

Neural input is important for mediating normal salivary gland secretion and salivary glands are innervated by both parasympathetic and sympathetic nerves. The primary neurotransmitters that mediate fluid secretion are acetylcholine and adrenaline and to a lesser extent neuropeptides (Proctor and Carpenter, 2007). Neuropeptides including calcitonin gene-related peptide (CGRP) and substance P (SP) target the main and small ducts as well as blood vessels. They are located in the sensory nerve fibers, which are carried by the parasympathetic and sympathetic nerve bundles (Kobashi et al., 2005; Ferreira and Hoffman, 2013) . It is known that CGRP coexists with SP in sensory neurons, which is present in all major salivary glands. The highest concentration of CGRP was found in the submandibular glands, and the highest concentration of SP was found in parotid and submandibular glands (Ekstrom et al., 1988). Both neuropeptides are involved in salivary secretion in rats and CGRP plays a role in the secretory response of SP (Ekstrom et al., 1984; Ekstrom et al., 1987; Ekstrom et al., 1988). In humans, levels of CGRP and SP are higher during salivary stimulation (Dawidson et al., 1997).

Atropine inhibits salivary secretion by blocking muscarinic receptors and suppressing the release of SP (Yu et al., 1983). There are other neuropeptides such as substance K and vasoactive intestinal peptide (VIP), which are also involved in evoking salivary secretion (Ekstrom et al., 1983; Ekstrom, 1987). Here, only CGRP and SP were briefly explained because they were selected and included in this study.

The role of parasympathetic nerves in fluid secretion through releasing acetylcholine led to the use of botulinum toxin for treating hypersalivation from nerves (Cordivari et al., 2004; Patel and Hoffman, 2014). This toxin blocks the release of acetylcholine (Cordivari et al., 2004). The use of botulinum has inhibited salivary secretion in cats resulting in xerostomia (Dickson and Shevky, 1923). Unlike atropine, botulinum toxin has a lasting effect and is thought to be a possible treatment for hypersalivation when using the appropriate dose (Bushara, 1997).

Botulinum toxin was initially used for treating strabismus and dyskinesias of skeletal muscles as well as Frey's syndrome (Scott, 1981; Laskawi et al., 1994; Drobik and Laskawi, 1995).

Botulinum toxin is a 150 kDa metalloprotease, which is produced by *Clostridium botulinum* and comprises of heavy and light chains (Figure 5.1) (DasGupta and Sugiyama, 1972; Simpson, 1980). There are eight immunological serotypes of botulinum: A, B, C1, C2, D, E, F and G (Blitzer and Sulica, 2001).

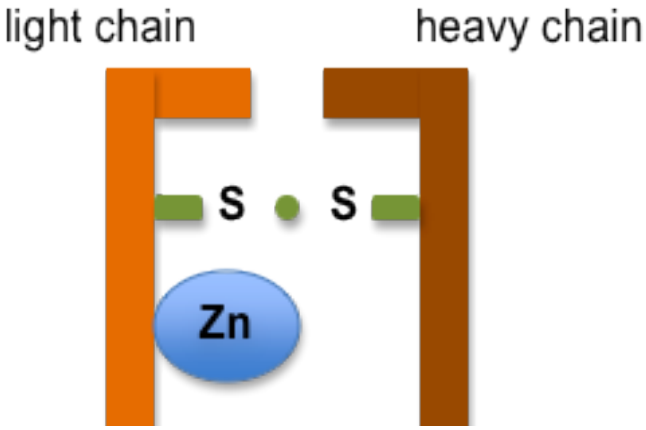


Figure 5.1 Botulinum toxin structure. It is composed of light chain bound to one zinc atom and a heavy chain with a disulfide bridge [Modified from (Brin, 1997)].

Botulinum toxin type A (BonT/A) is most commonly used among the different serotypes because it is able to cleave synaptosomal-associated protein (SNAP-25) subunits (Brin, 1997). The mechanism of action of the neurotoxin relies on two main steps. The first step is the parent cleavage of the chain resulting in a heavy chain bound to a disulfide bond (around 100 kDa) to a light chain that is bound to one atom of zinc (around 50 kDa) (Schiavo et al., 1992). Three steps are then required for mediating paralysis: 1) internalization, 2) reduction and translocation of the disulfide to the cytosol and 3) the inhibition of neurotransmitters release (Brin, 1997).

In the case of salivary glands, the action of botulinum relies on inhibiting the release of presynaptic acetylcholine (Sollner et al., 1993). Thus, a depression of the parasympathetic-dependent secretory function occurs as a consequence of the acetylcholine inhibition (Capaccio et al., 2008). It also can modify the concentrations of enzymes and solutes of saliva such as amylase (Ellies et al., 2002; Ellies et al., 2004).

In the previous chapter, ligation/de-ligation appeared to affect the culture of salispheres *in vitro*. In this study, the inhibition of the neural input *in vivo* and *in vitro* was included to determine the role of neural influence during salisphere development, and most importantly, to explore whether neural aberration would lead to similar effects to grown salispheres from injured glands.

5.2 Materials and methods

5.2.1 Experimental design

The experiments were divided into two categories; *in vivo* and *in vitro* experiments.

In vivo: Mice were anaesthetized, as described previously, with ketamine/xylazine and injected with 0.5 U of BoNT/A into the right Wharton duct leading to the submandibular gland. The left glands (contralateral glands) were injected with saline. Seven days later, under terminal anaesthesia, the submandibular glands were dissected, weighed, and processed for protein analysis, morphometric assessments and salispheres culture. The diagram below summarizes the experiments that have been carried out in this chapter:

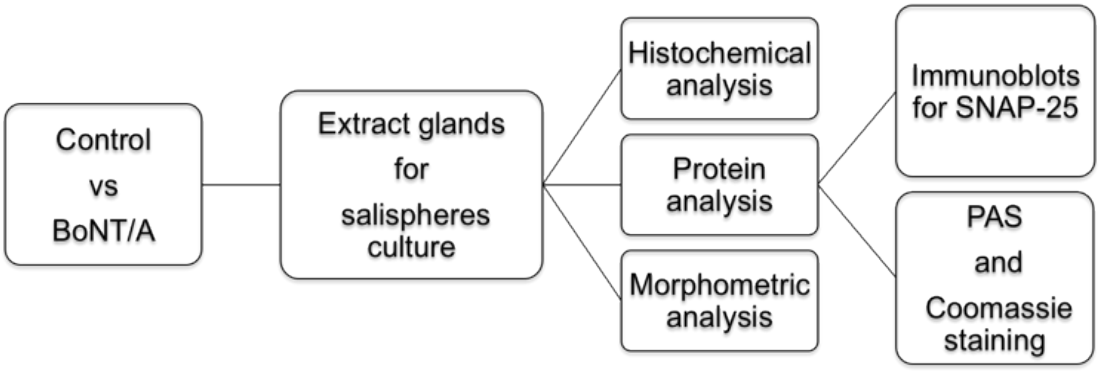


Figure 5.2 Diagram showing the experimental design for the *in vivo* study of BoNT/A injections.

In vitro: Salispheres were cultured with different neuropeptides (SP and CGRP) and atropine. The morphology of salispheres and their protein expression, were

assessed at different time-points. Figure 5.3 shows the experimental design for the *in vitro* experiments:

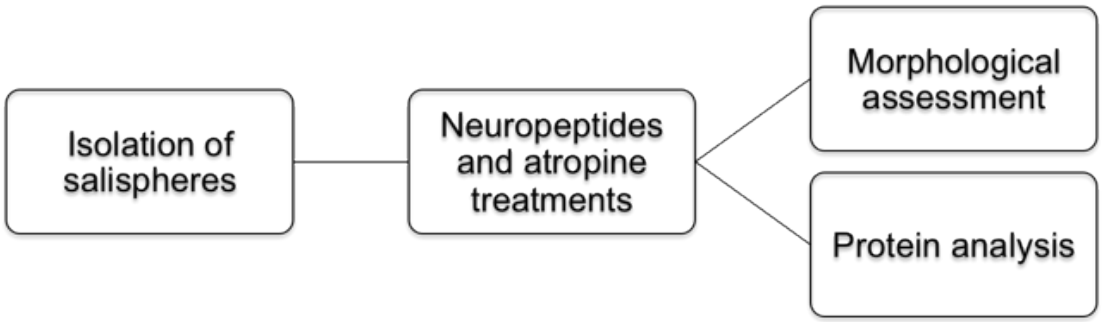


Figure 5.3 A diagram illustrating the *in vitro* experimental plan for salispheres treatment with different neuropeptides and atropine.

5.2.2 BoNT/A injections

For BoNT/A experiments, cannulation of the submandibular glands via the Wharton duct was preformed according to Kurki et al. (2011) protocol. The mouse was firstly anesthetized with xylazine (5 mg)/Kg)/ketamine (25 mg/Kg) i.p injections and positioned ventrally. Then, the maxillary incisors were locked by a metal wire and the mandibular incisors were hooked by an elastic string in order to keep the mouth open (Figure 5.4).

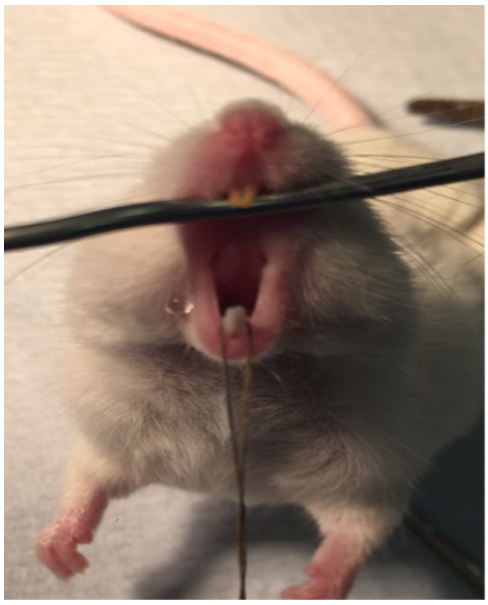


Figure 5.4 The cannulation of the submandibular gland duct. The cannulation of the duct requires locking the maxillary incisors by a metal wire and hooking the mandibular incisors by an elastic string.

Next, an insulin syringe with a 29G needle was placed into a 0.58 diameter polyethylene tube. The polyethylene tube was inserted approximately 5 mm inside the duct.

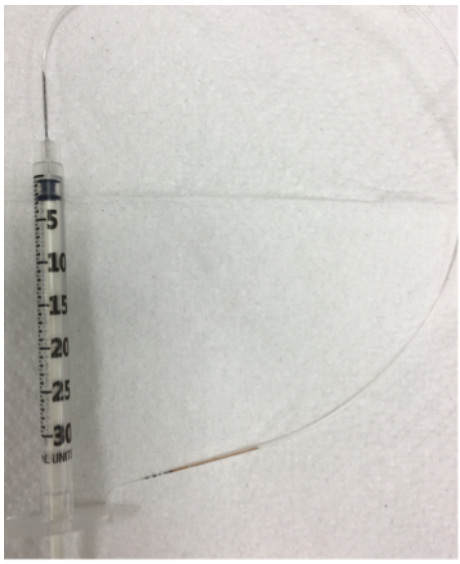


Figure 5.5 An image of a 0.58 diameter polyethylene tube placed into a 29G needle of an insulin syringe.

An injection of 0.5 U of BoNT/A (Allergan, USA) in 50 µl of saline containing around 2 µl of trypan blue was injected into the duct of submandibular gland. Pressure was retained on the piston for few minutes after injection, to avoid any backflow of the infusion. The mouse was kept in the incubator and was monitored until it recovered from the anaesthesia. Seven days post-injection, mice were terminally anesthetized with an overdose of pentobarbitone. The submandibular glands were dissected and weighed before further analysis.

5.2.3 Isolation and culture of salispheres from BoNT/A injected glands

The isolation and culture of salispheres from BoNT/A treated glands were carried out according to the protocol in section 2.2, and glandular weights were taken into consideration as only one gland was used for BoNT/A injections. The MTS viability assay was performed at day 4 of culture as described in section 2.10. In addition, the number and size of salispheres were counted, and the size of the salispheres was measured at day 4, as described previously in sections 2.3 and 2.5. Salispheres were collected at day 4 of culture as explained in section 2.6.

5.2.4 Neuropeptides and atropine *in vitro* treatments

Cells were directly treated prior to culture with CGRP, SP and atropine. Table 5.1 shows the concentrations of CGRP, SP and atropine used in the salisphere culture media, which concentrations were based on previous literature and preliminary experiments.

Treatment	Concentration
CGRP	1 μM
SP	10 μM
Atropine	10 μM

Table 5.1 Concentrations of neuropeptides and atropine applied in 1 ml of culture media.

At day 4 and 8 of culture the salispheres were counted and the size of salispheres was measured, as described in sections 2.3 and 2.5.

5.2.5 Gel electrophoresis and immunoblotting

All salispheres and tissue samples were separated by SDS-PAGE and run under reducing conditions. Equal amounts of protein were loaded for tissue samples, whereas equal volumes of salisphere samples were loaded for gel electrophoresis.

5.2.6 Antibodies

Antibodies	Abbreviation	Dilution	Company
Cleaved Synaptosome Associated Protein	Cleaved SNAP-25	1:1000	Cell signaling, UK
P44/42 MAPK (ERK1/2)	p-ERK	1:1000	Cell signaling, UK

Table 5.2 Antibodies which have been used in chapter 5

All Immunoblots were probed overnight including p-4e-bp1 and p-S6 rp antibodies as described previously in section 2.8.

5.2.7 Tissue collection and homogenization

Tissues were collected and homogenized as previously described in sections 2.12 and 2.13.

5.2.8 Protein concentration quantification

Protein concentrations of controls and BoNT/A treated tissue homogenates were quantified by the BCA assay as explained in section 2.14.

5.2.9 Periodic Acid Schiff's staining and Coomassie Brilliant staining

Gels were processed for Periodic Acid Schiff's staining and Coomassie Brilliant staining as described in sections 2.15 and 2.16.

5.2.10 Tissue sectioning and histochemical staining

The procedure of tissue sectioning and histochemical staining of controls and BoNT/A treated submandibular glands were carried out according to the protocol described in section 2.17.

5.2.11 H&E staining

Tissue sections were morphologically assessed by H&E staining as explained in section 2.18.

5.2.12 Immunohistochemistry

Tissue sections were stained with Caspase-3 (R&D systems, UK) antibody at a dilution of 1:300, to investigate the presence of apoptosis after BoNT/A treatment. The staining protocol is explained in section 2.20.

5.2.13 Statistical analysis

For BoNT/A experiments, unpaired two tail t-test was performed. Data acquired from the *in vitro* experiments were analyzed by one-way ANOVA followed by Tukey's multi-comparison test, as described in section 2.21.

5.3 Results

5.3.1 Glandular weights

After seven days of BoNT/A injections, glandular weights were directly measured after dissecting the glands. Statistically, there was no difference between the glandular weights of BoNT/A injected glands (57.75±3.52, n=4) and the glandular weights of controls (58.81±0.47g, n=4) (Figure 5.6).

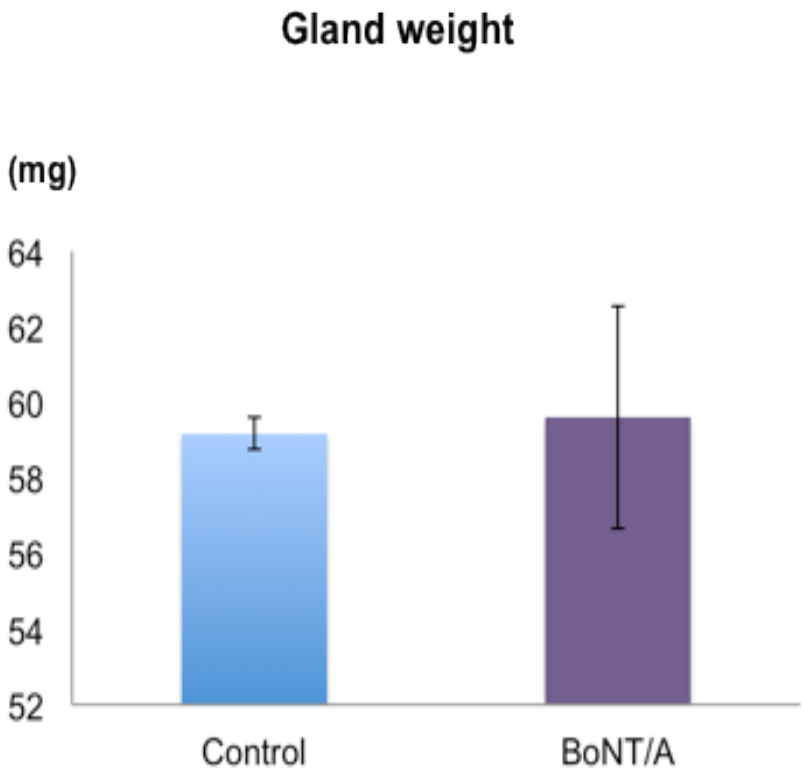


Figure 5.6 Mean gland weights. The mean gland weights of control glands and BoNT/A injected glands showed no significant changes (p=0.3875) (mean±SEM, n=4).

5.3.2 Cleavage of SNAP-25 demonstrated successful drug delivery to the glands

The use of cleaved SNAP-25 antibody helped in determining the action of BoNT/A in the glands. A visible second band appeared (Figure 5.7) only in BoNT/A treated glands whereas only one band appeared in the control.

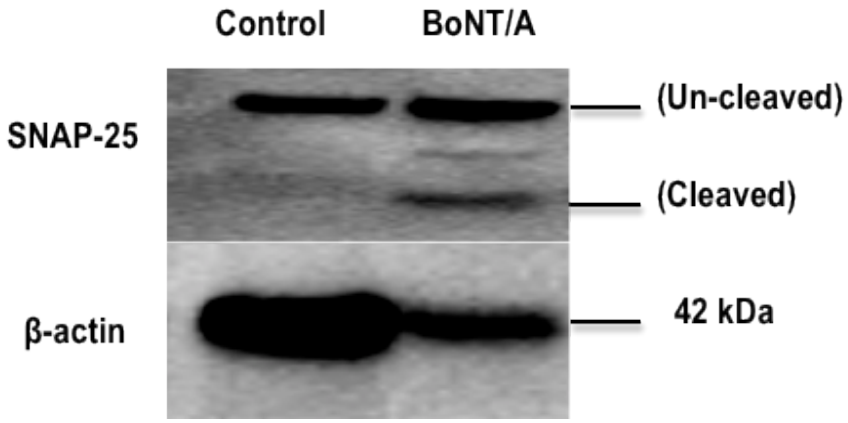


Figure 5.7 Cleaved SNAP-25 expression levels in control and BoNT/A injected glands. The second band represents cleavage of SNAP-25, which occurred only in BoNT/A injected glands, n=4.

5.3.3 Effects of BoNT/A injections biochemical and histological analysis

To determine the effects of BoNT/A on the glands, mucin levels were measured by the PAS staining. BoNT/A treated glands showed similar levels of mucins to controls (Figures 5.8, A and B). In addition, total protein levels analyzed using Coomassie staining, were also similar to non-treated glands (Figure 5.8, C).

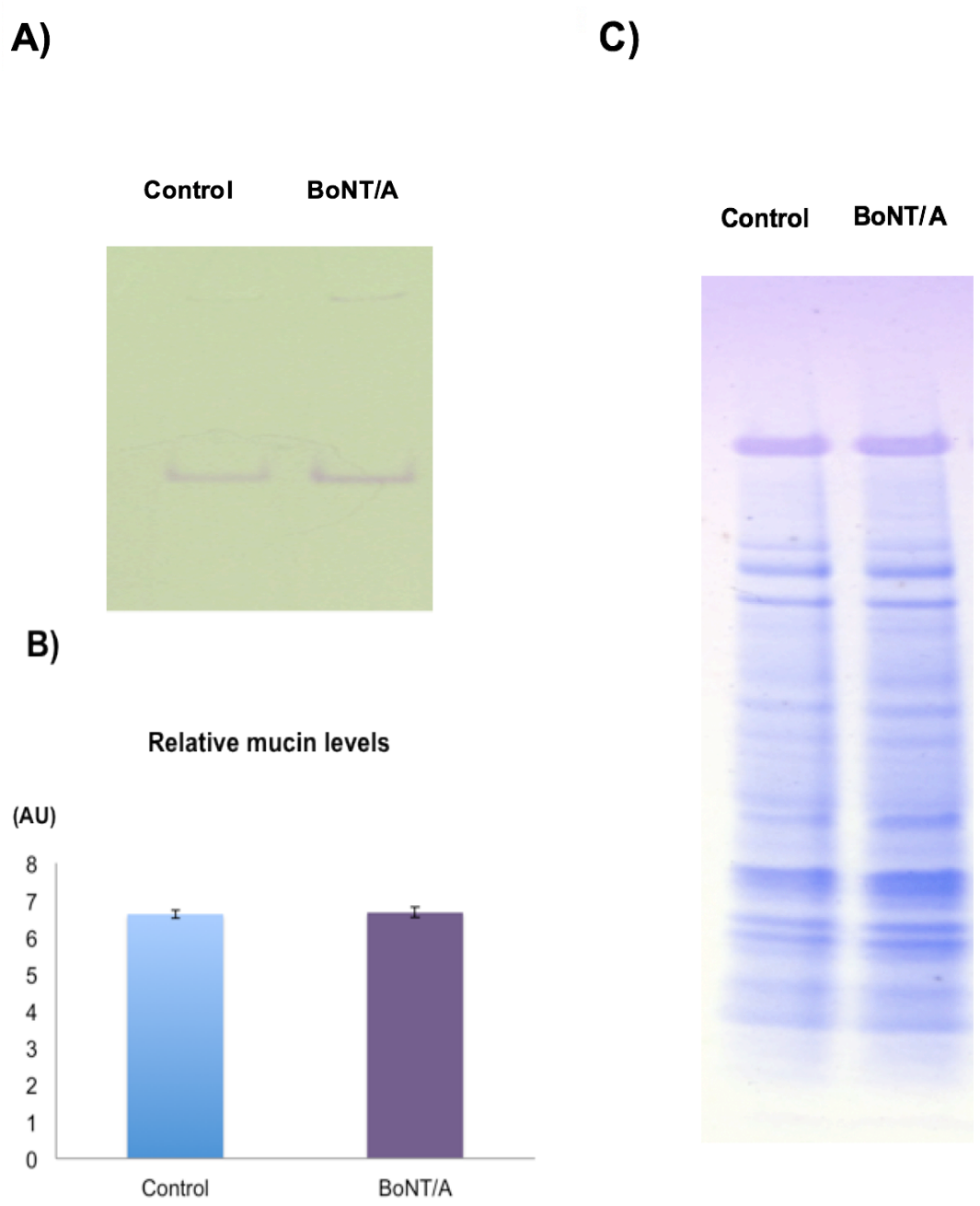


Figure 5.8 Mucin and total protein levels from BoNT/A treated submandibular glands. A) Periodic Schiff's staining and B) Coomassie staining of gland homogenates of untreated glands and seven day treated submandibular glands with 0.5U BoNT/A. C) Relative levels of mucins after BoNT/A treatment appeared similar to untreated glands (mean±SEM, n=3).

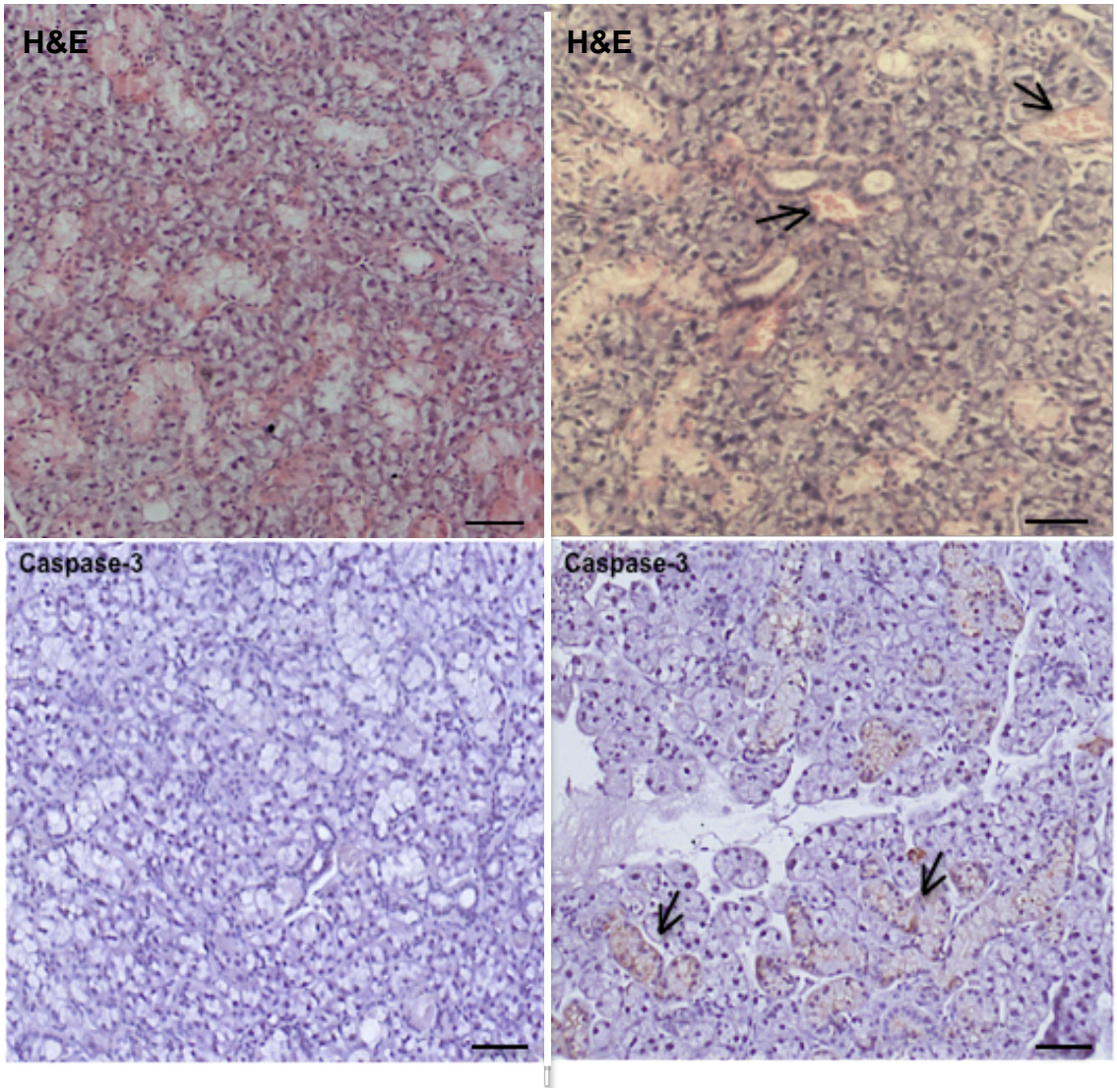


Figure 5.9 H&E staining and immunohistochemistry of caspase-3 and of untreated and BoNT/A treated submandibular glands. Upper panel indicates histological appearance of acinars and ducts of control (untreated glands) and BoNT/A injected glands. BoNT/A injections did not show distinctive effects on histology of submandibular glands, except some enlarged blood vessels were observed post-injection (black arrow). Lower panel indicates immunohistochemistry of control (untreated) and of BoNT/A treated tissue sections which showed weak staining against caspase-3 in BoNT/A treated glands and mostly appeared in (black arrow) ducts, whereas control glands were almost negative against capsase-3. The scale bar represents 20 µm.

In addition, acinars and ducts appeared unaffected by BoNT/A as H&E staining showed similar morphology to controls. However, immunohistochemical staining of caspase-3 demonstrated some apoptotic bodies in BoNT/A treated glands. Although, the staining was weak, caspase-3 was mostly detected in the ducts (Figure 5.9).

5.3.4 The action of BoNT/A did not abolish salispheres formation *in vitro*

Isolating and culturing salispheres from injected glands with BoNT/A, showed similar behavior of cultured salispheres from a normal healthy gland. Both, MTS levels ($p=0.2624$) (Figure 5.10, B), number of salispheres ($p=0.1020$) (Figure 5.10, C) and size of salispheres (Figure 5.10, D) at day 4 of culture exhibited no statistical differences compared to controls. Interestingly, BoNT/A injections caused the salispheres to adhere by day 6 of culture, but no fibroblastic like structures were observed.

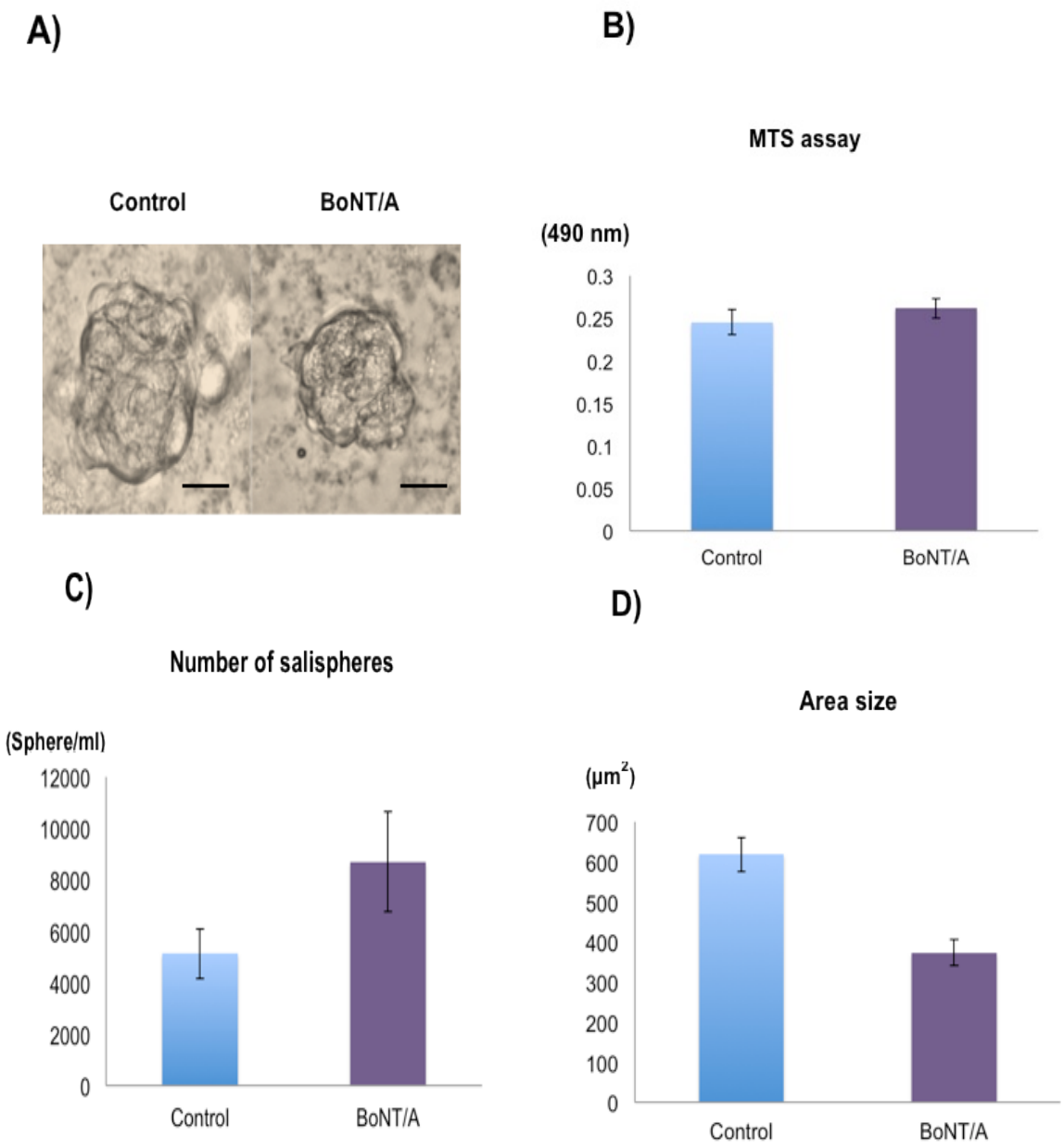


Figure 5.10 Effects of BoNT/A on culture of salispheres. A) Images of developing salispheres at day 4 of culture from untreated glands and those derived from gland injected with BoNT/A. B) Absorbance values of MTS viability assay showed no impact occurred post BoNT/A injections. C) Salispheres numbers were comparable to controls as well as D) area size of salispheres. Bars represent mean± SEM, n=3.

5.3.5 Effects of neuropeptides and atropine showed on salispheres culture

To evaluate the effects of neural input and inhibition *in vitro*, salispheres were cultured from day 0 with the neuropeptides CGRP and SP, as well as atropine, which is an inhibitor of parasympathetic cholinergic signalling.

Generally, the neuropeptide treatment did not affect the formation of salispheres or affect their culture at days 4 and 8 of culture, as the number and size of the salispheres were comparable to controls. However, only atropine caused a significant decline in number of salispheres at day 4 of culture (Figures 5.11, A, B and C). Conversely, mTOR expression appeared unaffected by the treatments as the phosphorylation of 4e-bp1 and S6 rp was comparable to the untreated salispheres (Figure 5.12).

As p-ERK is associated with the activation of CGRP and SP, the phosphorylation of ERK was measured at day 8 of salisphere culture, treated with neuropeptides and atropine. The expression of p-ERK clearly appeared in SP treated salispheres, but was abolished by atropine treatment (Figure 5.13).

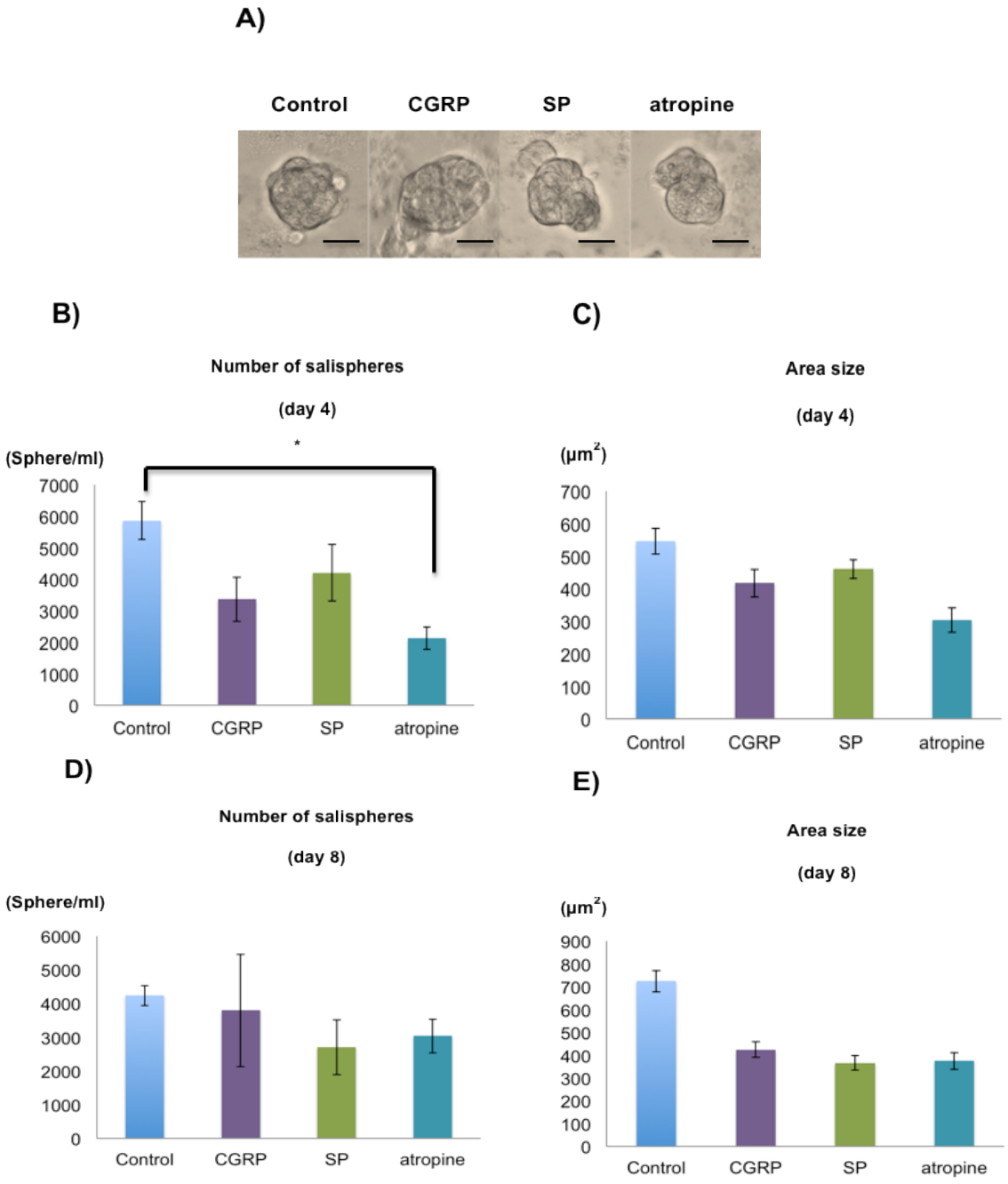


Figure 5.11 Morphological assessment of salispheres after 4 and 8 days of culture with CGRP, SP and atropine. A) Images of salispheres at day 4 of culture in the presence of neuropeptides and atropine (*p<0.05). B) Salisphere numbers were significantly reduced after atropine treatment. C) Area size of all cultured salispheres was comparable to controls. D) The number of atropine treated salispheres recovered by day 8 of culture. E) However, the mean area size of neuropeptide and atropine treated salispheres was significantly decreased at day 8 of culture (*p<0.0002). Bars represent mean± SEM, n=3.

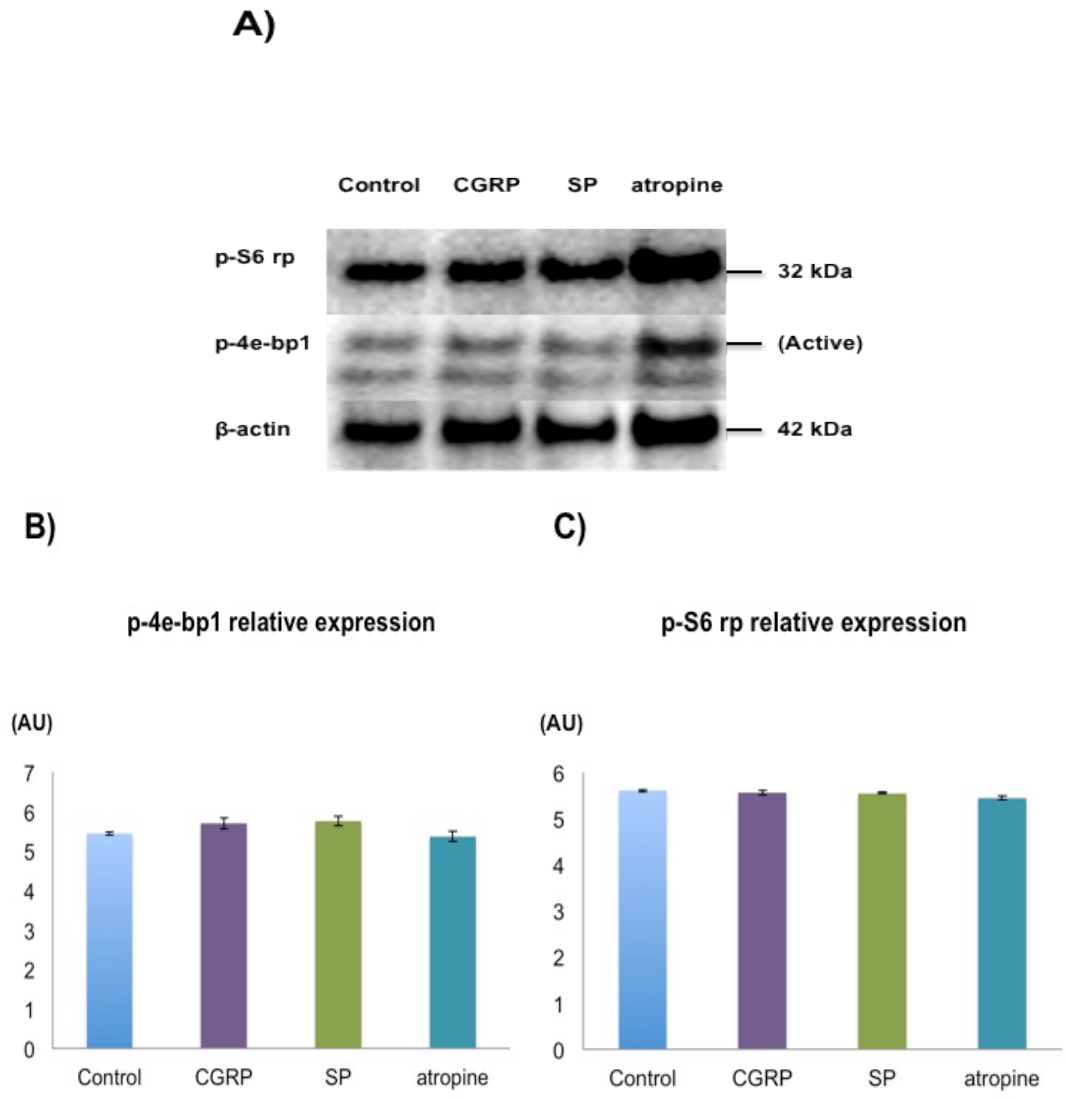


Figure 5.12 mTOR levels in neuropeptide and atropine treated salispheres at day 4 of culture. A) Immunoblot of salisphere lysates probed with p-S6 rp, p-4e-bp1 and β-actin. Both the B) relative expression of 4e-bp1 and C) S6 rp reflect no differences in mTOR expression in the presence of either neuropeptides or atropine. Bars represent mean± SEM, n=3.

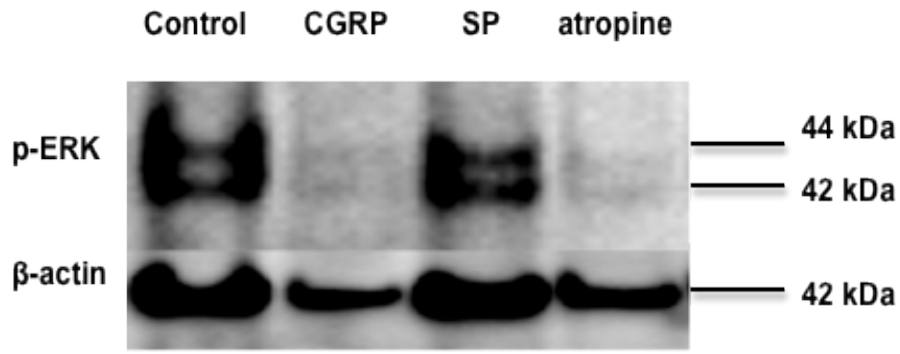


Figure 5.13 p-ERK expression in neuropeptide and atropine treated salisphere lysates. Blot represents of two experiments and presented as preliminary evidence only.

5.4 Discussion

BoNT/A is commonly used to reduce salivary secretion in hypersalivation disorders. In addition, BoNT/A has shown a protective role against radiotherapy-induced damage to salivary glands (Teymoortash et al., 2009). These experiments were designed to understand the effects of BoNT/A on gland and progenitor cell culture because BoNT/A is correlated with the suppression of salivary secretion. My objective in this chapter was to correlate the effects caused by injury on salispheres (Chapter 4), to the inhibition of neural input on to salispheres, to reveal how the autonomic nerve supply could be important for glandular regeneration (Osailan et al., 2006b).

The inhibition of salivary secretion by BoNT/A is time and dose-dependent (Xu et al., 2015). Most studies using rat and rabbit have applied BoNT/A by intraglandular injections (Ellies et al., 1999; Shan et al., 2013; Xu et al., 2015). In this study, the cannulation procedure was chosen to insure a uniform delivery of BoNT/A into the gland. The difficulty of this process was to avoid any backflow of the infusion due to the pressure.

In these experiments, the seven days injections of BoNT/A caused no difference on gland weights, similar to findings in rabbits (Figure 5.6) (Shan et al., 2013). Since BoNT/A directly affects the cleavage of SNAP-25, the detection of cleaved SNAP-25 is an indication of BoNT/A delivery and action (Figure 5.7). In rat experiments the cleavage of SNAP-25 at neuroglandular junctions occurred within seven days of injection (Xu et al., 2015). Interestingly, the SNAP-25 was also cleaved in the contralateral glands, suggesting that a leakage of BoNT/A might occurred., for this reason the contralateral glands were excluded from this study.

As SNAP-25 was cleaved by BoNT/A, further analysis was performed to detect possible biochemical and histochemical changes to the salispheres before and after BoNT/A administration. Protein analysis revealed that mucin and total protein levels, and histology, were unaffected by the toxin (Figures 5.8, A and B). However, cleaved SNAP-25 and evidence of apoptosis suggest that the injections affected the glands without causing atrophy (Figure 3.8, C) (Coskun et al., 2007).

Similar to these findings, previous investigations have shown that BoNT/A induces apoptosis, but other studies did not observe apoptosis nor changes in acinar cell volume or lymphatic infiltration (Figure 5.9) (Ellies et al., 1999; Ellies et al., 2000; Coskun et al., 2007; Shan et al., 2013). Presumably, the delivery technique used might affect the apoptotic level without causing morphological damage, because an intraglandular injection of a concentrated amount of BoNT/A, in a defined area, could cause more stress and toxicity.

In contrast to salivary gland atrophy, the ability to isolate and culture salispheres post BoNT/A injections suggest that suppressing parasympathetic innervation would not affect the progenitor pool. This observation also suggests that parasympathetic innervation acts differently on an irradiated or ligated glands. However, the adherence property of cultured salispheres occurred at day 6, suggesting that attachment was likely driven by minor parasympathetic inputs.

As parasympathetic nerves play a role in regeneration, *in vitro* experiments of CGRP and SP were also included (Proctor and Carpenter, 2007). During denervation of salivary glands, both CGRP and SP were down regulated around the salivary gland ducts (Gibson et al., 1984; Soinila et al., 1989). Moreover, ductal ligation causes

salivary gland atrophy and decreases SP release (Ekstrom et al., 1984). As sympathectomy causes an increase in CGRP and SP levels in both parotid and submandibular glands, *in vitro* experiments of CGRP, SP and atropine were included to investigate if neuropeptide stimulation and inhibition affected the characteristics of salispheres *in vitro* (particular adhering to the plastic dish).

The use of neuropeptides has not mimicked the action of BoNT/A treatment on salisphere formation. Although, statistically there were no differences due to the treatments, previous studies have shown that continuous stimulation of neurotransmitters can damage cellular function, which might explain the minor effects on salisphere size at a late stage of culture (day 8) (Horie et al.,1996). In addition, mTOR expression in treated salispheres was unaffected by neuropeptide or atropine treatment, emphasizing that mTOR is more likely to be responsible for salisphere growth.

Neuropeptides including CGRP and SP are associated with the activation of the extracellular signal-regulated kinase (ERK) (Parameswaran et al., 2000; Lallemend et al., 2003). In the present study, phosphorylation of ERK occurred in untreated salispheres, and the inhibition of ERK phosphorylation by atropine suggests that normal salispheres might contain endogenous neuropeptides. Under these conditions, SP showed a greater effect on ERK expression compared to CGRP treated salispheres, suggesting that the phosphorylation of p-ERK is principally stimulated by SP.

In conclusion, the *in vivo* and *in vitro* experiments suggest that neural suppression did not considerably affect the characteristics of salispheres. Although, the minor

effect caused by BoNT/A to form salispheres suggests that neural alterations might play a minimal role in altering the morphology of salispheres from injured glands.

6 TRANSPLANTATION OF MODIFIED SALISPHERES INTO SALIVARY GLANDS

6.1 Introduction

Radiotherapy can cause irreversible salivary dysfunction and loss. Specifically, irradiation negatively affects saliva flow rate, gland weight and acinar cell numbers (Burlage et al., 2001; Coppes et al., 2009). In addition, it causes cellular damage such as apoptosis, which occurs post-radiation (Vissink et al., 1990; Paardekooper et al., 1998).

Recently, stem cell transplantation into damaged salivary glands has been widely investigated as a method to rescue salivary glands from damage. In 2006, Lombaert et al. found that transplantation of BMCs into an irradiated gland was able to improve salivary function and morphology (Lombaert et al., 2006b). However, limited access to BMCs led to the discovery of salivary gland stem/progenitor cell transplantation (Lombaert et al., 2008a).

Different stem cell marker expressing cells such as CD49f, CD133, and c-kit have been transplanted into irradiated glands. All of these transplanted cell expressing markers have improved saliva production. However, the isolation and transplantation of c-kit expressing cells into irradiated glands has shown to be the most effective for gland recovery, compared to other stem cell expressing markers, which require higher cell numbers for successful regeneration (Lombaert et al., 2008a; Feng et al., 2009; Nanduri et al., 2013,). Conversely, ligation of the main excretory duct induces atrophy, which is characterized by apoptosis and acinar cell loss (Shiba et al., 1972; Takahashi et al., 2000). The removal of the obstruction allows the glands to regenerate and functionally recover. In other studies, morphological and cellular regeneration markers are upregulated after three days of de-ligation. The expression

of perinatal proteins in the immature acinar cells during de-ligation suggest regeneration occurs via an embryonic/prenatal pathway (Cotroneo et al., 2008).

The degree of glandular recovery is time-dependent on the length of ligation. For example, in rats, 8 weeks of de-ligation allows acinar cells to appear normal, yet the acinar cells were smaller than acinar cells of a normal gland (Osailan et al., 2006a). However, in other studies on other species showed that full recovery appeared after seven days of de-ligation (Bhaskar et al., 1966). It has been also previously reported that longer periods could result in full recovery of acinar cells (Tamarin, 1971a).

Growth factors and signalling pathways previously investigated which have shown to be involved in the protection of salivary glands against damage (Lombaert et al., 2008b; Hai et al., 2012; Hai et al., 2014). For this reason, it would be interesting translate the *in vitro* effects of several inhibitors such as rapamycin, LiCl and ROCKi to the *in vivo* behavior of salivary glands.

Indeed, salivary gland regeneration is a promising field for ameliorating radiation induced salivary gland damage. In this chapter, this possible treatment was explored by injecting treated salispheres with selected inhibitors, including rapamycin, LiCl, ROCKi, into normal salivary glands. These molecules and their combinations have shown exciting and distinctive effects on salisphere characteristics and therefore were selected for the following *in vivo* study.

Work has been performed so far on normal salivary glands to explore technical experimental approaches and to evaluate the physiological response of salivary glands to injections. In addition, intraglandular injections of treated salispheres have

been performed once in de-ligated glands to compare the ability of these injections of recovering damaged gland. Future studies on damaged glands would confirm whether these injections would encourage regeneration and the recovery process.

6.2 Materials and methods

6.2.1 Experimental design

In this chapter, normal submandibular glands were injected with salispheres that had been treated with selected inhibitors from previous experiments. Only one de-ligation experiment was performed for each salisphere injection. At the end of each experiment, glands were collected for biochemical and histochemical analysis.

6.2.2 Isolation and culture of salispheres

The isolation and culture were processed according the protocol described in section 2.2. The cells were treated with several inhibitors at day 0 of culture. Table 6.1 shows the concentrations of the inhibitors, which have been used in this chapter:

Inhibitors	**Concentrations**	
LiCl	10 mM	
Rapamycin and LiCl (Rapa+LiCl)	Rapa	22 nM
	LiCl	10 mM
ROCKi	10 μM	

Table 6.1 Concentrations of inhibitors which have been used for salisphere culture in this chapter.

6.2.3 Salisphere counting

At day 4 of culture, salispheres were counted as previously described in section 2.4. Around 1,000 spheres were used for the intraglandular injections.

6.2.4 Salisphere preparation

After four days of culture with the inhibitors, salispheres were incubated for 15 minutes with 1 µl of vybrant cell labeling solution. Salispheres were then pelleted at 400 g for 5 minutes and re-suspended with 200 µl of DMEM-F12 media.

6.2.5 Salisphere injections into normal and de-ligated submandibular glands

Mice were anaesthetized as previously descrived. A 0.5 cm skin incision was made in the centre of the neck. A blunt dissection was performed where the fat surrounding the salivary glands were dispersed and the salispheres were injected using a 26G needle into different areas of the glands. The mice were allowed to recover from the anaesthesia. After seven days, the mice were terminally anaesthetized with an overdose of pentobarbitone.

For salisphere injections into de-ligated glands, the submandibular gland ducts were ligated for seven days using a clip, skin was sutured and mice were allowed to recover from the anesthesia. Following seven days of ligation, de-ligation was processed for additional week. De-ligation and salisphere injections were carried out by the removal of the clip, followed by a direct injection of the salispheres into the glands. The skin then was sutured and the mice were allowed to recover from the anesthesia. Mice were terminally anesthetized after seven days of de-ligation.

At the end of all experiments the submandibular glands were collected and glands weights were measured before further analysis.

6.2.6 Tissue collection and homogenization

Tissues were collected and processed for homogenization as described previously in sections 2.12 and 2.13.

6.2.7 Protein concentration quantification

Protein concentrations were determined using the BCA assay as previously explained in section 2.14.

6.2.8 Immunoblotting

Equal amounts of proteins were processed for gel electrophoresis and immunoblotting as described in section 2.17.

6.2.9 Coomassie Brilliant Blue staining

Gels were used for protein staining with Coomassie Brilliant staining as described in section 2.16.

6.2.10 H&E staining and morphometric assessment

Tissue sections were stained with H&E as previously described in sections 2.18 and 2.19.

6.2.11 Statistical analysis

Statistical assessment was performed by one-ANOVA and Tukey's test as described in section 2.21.

6.3 Results

6.3.1 Gland weight assessment

The glandular injections of treated salispheres with rapamycin, LiCl, ROCKi, and co-treatment of rapamycin and LiCl resulted in slight, but statistically insignificant, increases in gland weights (p=0.2562) (Table 6.2) (Figure 6.1). In addition, no differences were observed in gland weights of regenerated glands caused by the injection, yet a slight increase was observed in injected regenerated gland with LiCl treated salispheres (Table 6.3) (Figure 6.5).

Intraglandular injections of treated salispheres with different inhibitors	**Gland weights (mg)**
Control glands	60.37 ± 2.02, n=3
Control injected glands	77.00 ± 10.17, n=3
LiCl injected glands	79.03 ± 6.46, n=3
ROCKi injected glands	70.87 ± 3.27, n=3
Rapa+LiCl injected glands	72.37 ± 2.82, n=3

Table 6.2 Mean weight of submandibular glands after seven days of Intraglandular injections with inhibitor-treated salispheres.

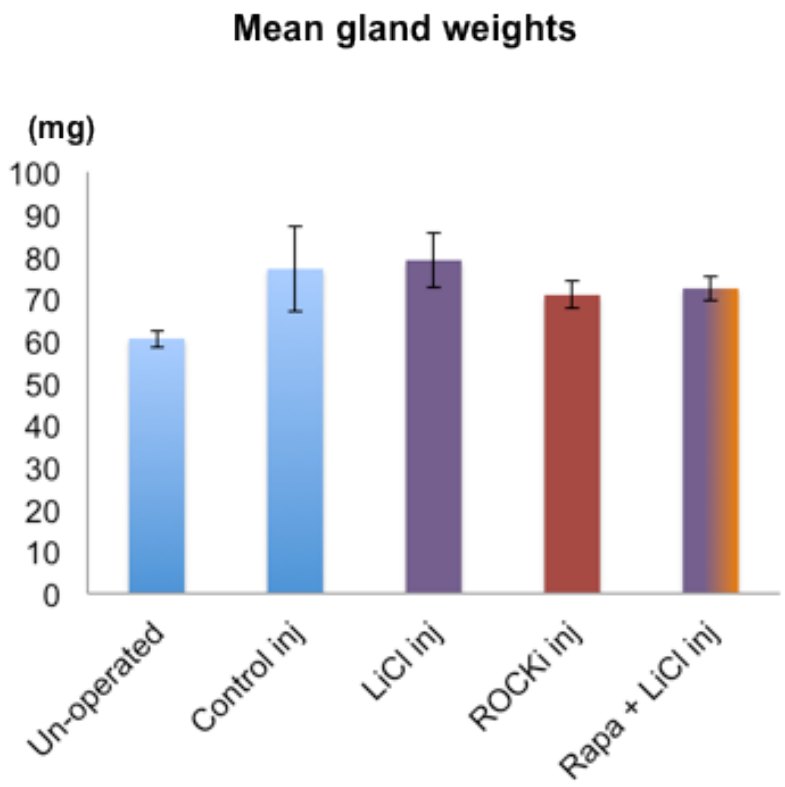


Figure 6.1 Mean submandibular gland weights. Injections of untreated salispheres (control inj). LiCl treated salispheres (LiCl inj), ROCKi treated salispheres (ROCKi inj) rapamycin and LiCl treated salispheres (Rapa+LiCl inj) into normal submandibular glands shows no significant effects on gland mass.

6.3.2 Coomassie staining showed some differences in total protein

Protein distribution among the different treatments was variable. The injection of treated salispheres with LiCl showed similar a protein pattern compared to the injection of untreated salispheres into the submandibular glands. While the injection of ROCKi treated salispheres and rapa+LiCl treated salispheres in submandibular glands demonstrated a more intense pattern of protein content compared to the other samples shown in Figures 6.2 and 6.5.

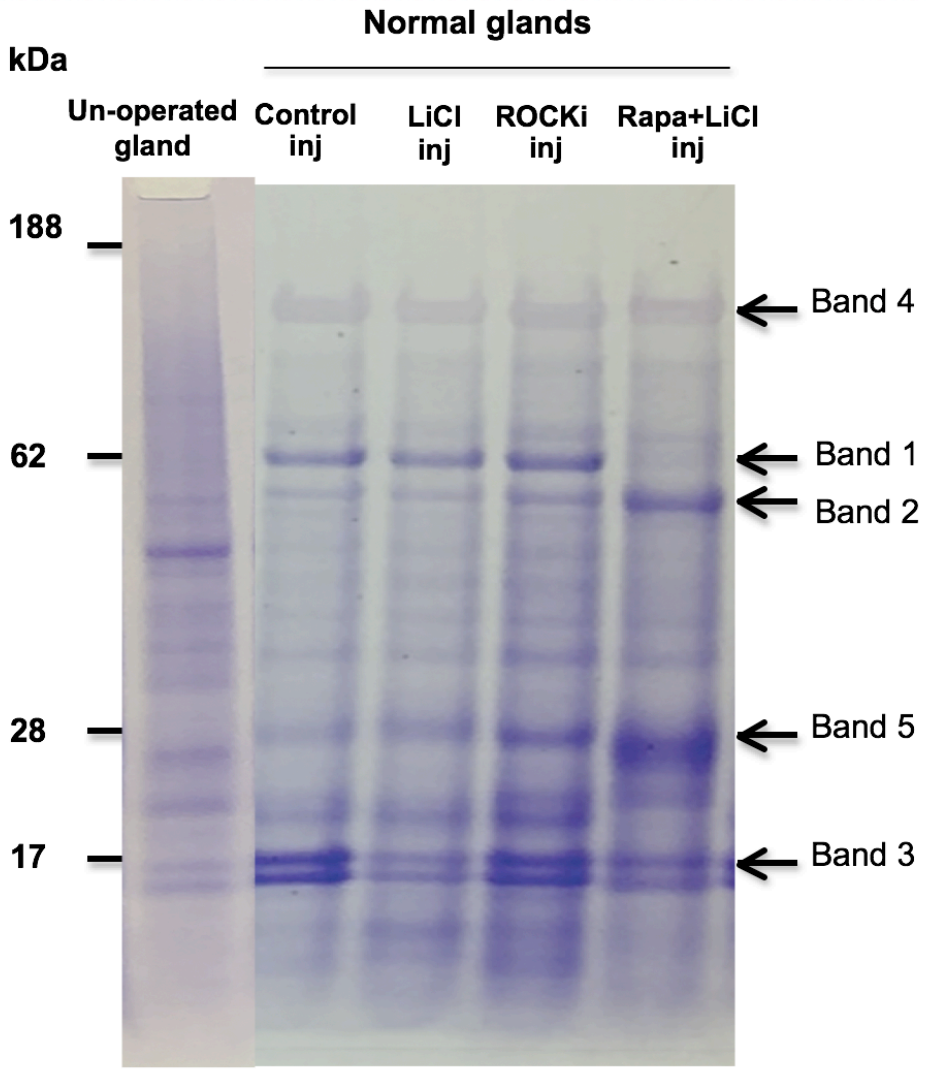


Figure 6.2 Total proteins detected by Coomassie blue staining of SDS-PAGE gels of submandibular gland homogenates. The ROCKi and rapa+LiCl showed a greater abundance of proteins compared to the control (band 3 and band 5), whereas minor mucin levels (band 4) appeared unchanged. Intranglandular injected glands with rapa+LiCl treated salispheres showed a complete lack of band 1.

6.3.3 Histological analysis post-treatment

Immunohistochemical analysis showed that injections did not cause significant damage to the glands. Neither injections of salispheres into normal glands (Figure 6.3) nor regenerated glands (Figure 6.7) affected their histology. Although injections of healthy salispheres showed a slight increase in acinar cell volume. However, the effects of all injections on acinar cell volume was insignificant (Figure 6.3) on both un-operated glands and regenerated glands (Figure 6.7).

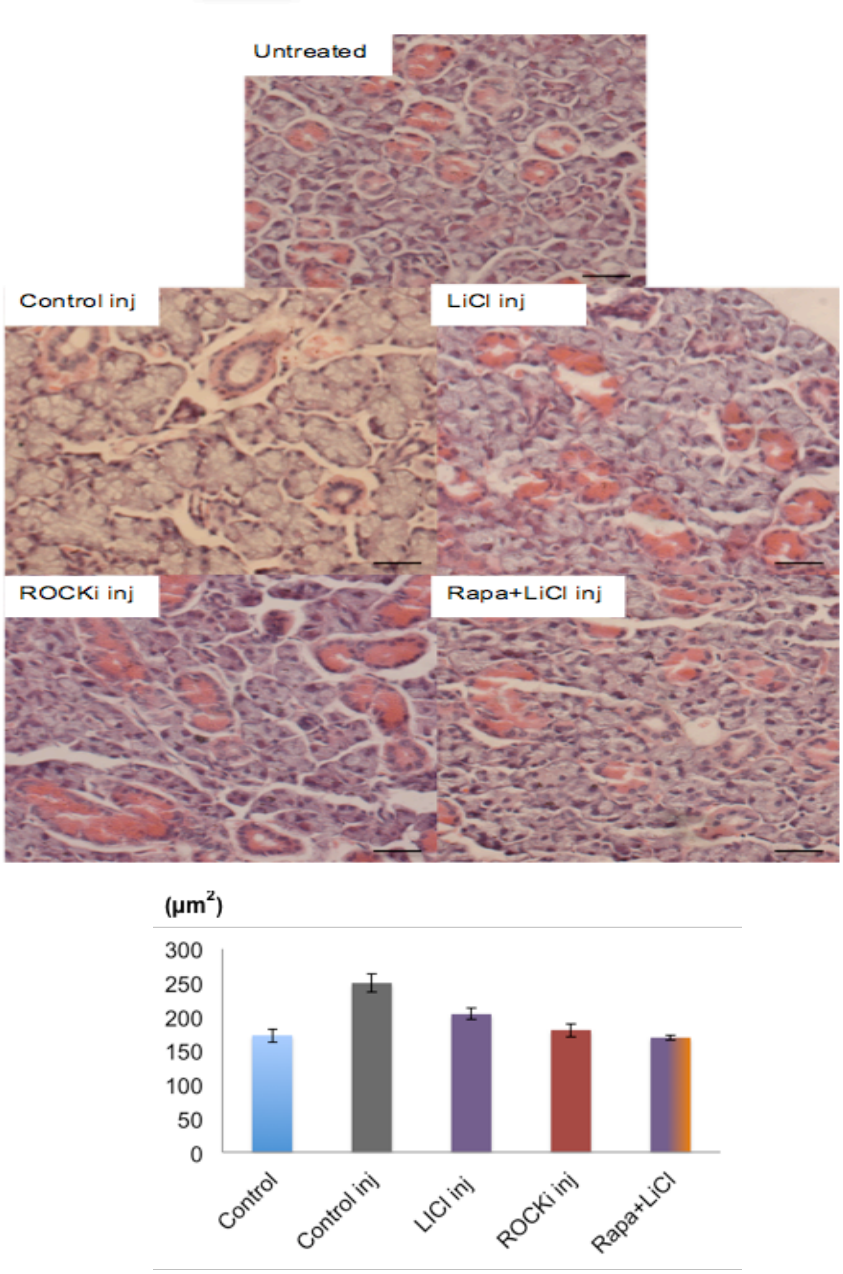


Figure 6.3 H&E and morphometric analysis of injected submandibular glands with salispheres. Images representing histological appearance of salivary glands after salisphere's intraglandular injections. Generally, no significant histological differences caused by the different treatments. The morphometric analysis showed a slight increase in the area size of the acini of injection with untreated salispheres and LiCl treated salispheres. The bar represents 20 µm.

6.3.4 Salispheres injections induce mTOR activation

To determine if mTOR is activated due to the intraglandular injections of salispheres into normal glands, immunoblots of tissue homogenates were probed with p-4e-bp1 and p-S6 rp. The injections of treated salispheres demonstrated that p-4e-bp1 is expressed in all samples. However, the phosphorylation of S6 rp only appeared in injected glands with ROCKi treated salispheres (Figures 6.4, B and 6.8).

Conversely, the intraglandular injections of untreated salispheres, LiCl treated salispheres, ROCKi treated salispheres and the co-treatment of rapa+LiCl salispheres into a regenerated gland showed an expression of S6 rp in all samples except 4e-bp1, which was only hyper-phosphorylated in injected glands with ROCKi treated salispheres.

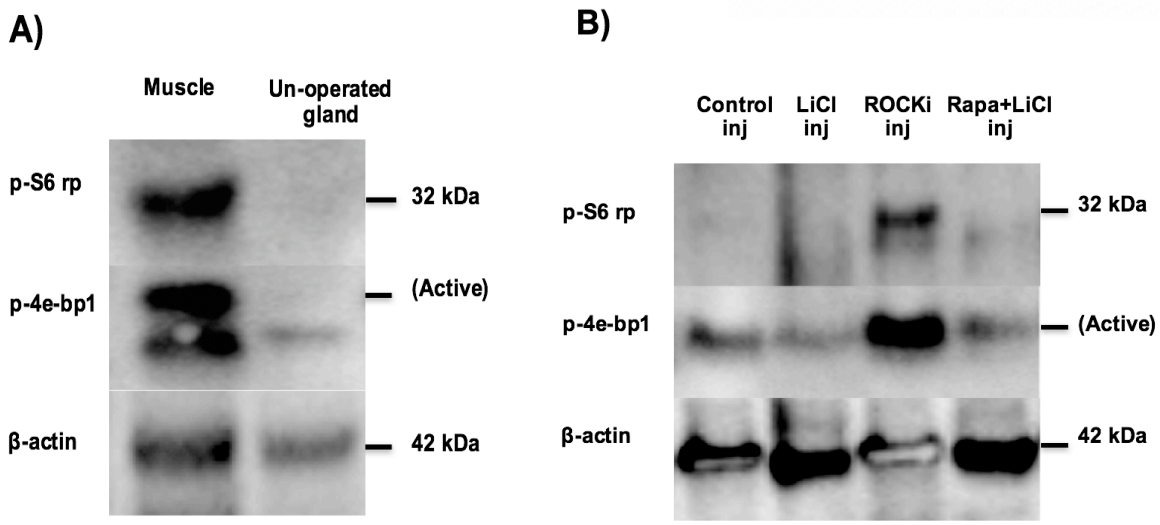


Figure 6.4 Effects of intraglandular injections of salispheres on mTOR activity. A) mTOR status in muscle from mouse biceps fermoris (positive control) and in mouse submandibular gland (negative control). B) Intraglandular injections appeared to phosphorylate 4e-bp1, while only ROCKi to affected both mTOR substrates, n=3.

6.4 Discussion

In vitro experiments of cultured salispheres with several inhibitors showed distinctive effects on salispheres culture including salisphere survival and adhesion. The injection of *in vitro* treated salispheres with several inhibitors into normal salivary glands was firstly performed to explore their physiological response to different injections.

Previous studies have used dissociated cells rather than salispheres for intraglandular transplantation of salivary gland progenitor cells (Lombaert et al., 2008a; Nanduri et al., 2013). However, in these experiments, salispheres were injected directly into the glands without dissociating them into single cells. The purpose for applying this method was to avoid any treatment attenuation upon salisphere dissociation. Another, reason was that expressing cell specific markers such as c-kit was not taken into consideration.

In addition, the effects of intraglandular injections of salispheres into de-ligated glands were determined (shown in Appendix A). This would help in exploring if any of these inhibitors could assist the gland recovery. However, this preliminary experiment was carried out only once due to pressure and atrophy caused by injury. Further optimization is required to minimize the variability caused by the injection and injury.

Although a slight increase in gland weight was observed post-injection, intraglandular injections of treated salispheres into normal submandibular glands the physiology of the glands, that is, the protein content, size of acini and histology appeared normal. This suggests that the injections generally did not negatively affect

the gland. However, the injection of LiCl treated salispheres into a regenerated gland resulted in the glandular weight recovery, because the gland weight was comparable an un-operated gland. A previous study demonstrated that glandular recovery increases by 50% after seven days of removal of obstruction. This suggests that the injection of LiCl treated salispheres into an injured gland could enhance the recovery by 30% compared to untreated de-ligated glands (Cotroneo et al., 2010).

LiCl is a GSK-3 inhibitor, which is involved in regulating both mTOR and Wnt. Normal homeostasis of haematopoietic stem cells requires mTOR and Wnt, where Wnt is involved in self-renewal and mTOR is involved in differentiation, thus regulating normal homoeostasis (Huang et al., 2009). This suggests that LiCl acted on both mTOR and Wnt resulting in *in vivo* recovery via the asymmetric division of the stem/progenitor pool (Coppes et al., 2009). This also suggests that the mechanism of action of LiCl *in vivo* is Wnt mediated, because a study showed that Wnt induced in progenitor cells transplantation helped in the recovery of normal homoeostasis (Maimets et al., 2016). However, functional analysis is required to confirm these findings.

In normal conditions, salivary glands undergo tissue cell loss due to aging, for this reason compensation is required to maintain tissue homeostasis. The compensation includes proliferation and differentiation of the progenitor cells (Coppes and Stokman, 2011).

Interestingly, mTOR activation in injected glands implies that signalling alterations occurred due to the injection, as all samples (salispheres injection into normal and regenerated glands) appeared to express either S6 rp or 4e-bp1. However, the

phosphorylation of both substrates of mTOR after the injection of ROCKi treated salispheres might indicate that ROCK is associated with mTOR and atrophy.

In conclusion, the effect of LiCl on a regenerated gland suggests that it might be a good tool for glandular recovery through salispheres. In addition, the effect of ROCKi on activating both mTOR substrates in normal glands and a regenerated gland suggest that there is a correlation between mTOR and ROCK. However, further investigations are required to discover the effect of these injections on glandular recovery post-injury. Moreover, further analysis is required to understand the mechanism, the safety of these injections and to confirm if LiCl could support glandular recovery.

7 DISCUSSION

7.1 Conclusions

The characteristics of salivary gland stem/progenitor cells have been well established by LRC and regeneration studies, proving their existence in the ductal compartment (Denny et al., 1993; Man et al., 2001; Kimoto et al., 2008), and highlighting the importance of the stem/progenitor pool for normal salivary gland tissue homeostasis (Pringle et al., 2013).

In addition, they are a promising therapeutic tool for repairing salivary glands after damage. Regeneration studies of salivary glands include ligation/de-ligation or transplantation of salivary gland stem/progenitor cells into an irradiated gland, demonstrated the ability of salivary gland to regenerate (Cotroneo et al., 2010). Transplantation of cells expressing specific stem cell markers such as c-kit into damaged glands clearly illustrated the regenerative potential of salivary glands (Lombaert et al., 2008a; Feng et al., 2009; Pringle et al., 2016; Nanduri et al., 2011). Additionally, the administration of growth factors or the activation of specific intrinsic signalling networks such as KGF and Wnt, have also shown a role in stimulating glandular repair post-radiation (Lombaert et al., 2008b; Hai et al., 2012).

Signalling networks are involved in numerous cellular processes. Specifically, the mTOR signalling pathway is involved in several roles such as protein synthesis, cell proliferation and differentiation (Laplante and Sabatini, 2012a) The translational machinery is regulated by mTOR and as previously stated, the cooperation of 4e-bp1 and S6 rp are responsible for initiating translation, and subsequently regulating cell growth and protein synthesis in both normal and disease states (Howell et al., 2013). One of the main objectives of this project was to explore the *in vitro* effects of mTOR inhibition on salisphere growth and culture. Another objective was to

investigate the *in vivo* response of salivary glands to intraglandular injections of inhibitor-treated salispheres on glandular recovery. In culture, the salivary gland stem/progenitor population pool is limited, and therefore a further objective was to determine if mTOR stimulation would increase the number of salispheres. Therefore, assessing mTOR activity in developing salispheres was an essential step before understanding the differences between growing salispheres from normal and injured glands.

Several upstream factors promote mTORC1 complex activity, including growth factors and insulin (Martin and Hall, 2005; Huang and Fingar, 2014,). The binding of EGF to cell surface tyrosine kinase receptors subsequently activates mTOR through the MAPK kinase (Huang and Fingar, 2014). In contrast to normal salivary glands, which lack mTOR activity, mTOR is activated during salisphere development from both normal and injured ligated glands. The activation of mTOR in salispheres suggests that the growth media contains sufficient insulin and growth factors to activate mTOR. In addition, data from rapamycin experiments demonstrated the necessity of mTOR for salisphere growth and survival, and subsequently their regeneration (Fingar et al., 2002).

Compensatory hyperplasia during ligation is accompanied with ductal and acinar cell proliferation (Walker and Gobe, 1987). In this present study, the increased number of salispheres in contralateral glands during ligation may be a compensatory hyperplasia, driven by the stem/progenitor cell population. Although, the mechanism of contralateral hyperplasia is unknown, changes in neural innervation may be implicated.

Cross talk between mTOR and Wnt was demonstrated by the action of LiCl on salisphere formation and development. *In vitro* experiments showed exciting effects of LiCl on salisphere morphology including size increases and the prevention of branching within the collagen/matrigel matrix. It is hypothesized that in a similar culture system, the size of neurospheres reflects their developmental maturity in response to growth factors such as EGF and FGF, as well as the proliferation and differentiation status of these stem/progenitor cells (Suslov et al., 2002). However, the LiCl treated salispheres preserved their spherical structure *in vitro*, suggesting that the stem/progenitor cells were too naïve to respond to EGF/FGF signals. Only by *in vivo* transplantation might help in assessing the regeneration significance.

Organoid formation is an indicator of successful differentiation as shown in previous studies and in this study (Feng et al., 2009; Maimets et al., 2016; Lombaert et al., 2017). As normal healthy salispheres are able to form acinar-ductal like structures in a collagen/matrigel matrix, this suggests that mTOR may be responsible for this process. Also, LiCl-treated salispheres preserve their spherical clusters, which implies that LiCl through stimulating Wnt prevents the formation of acinar-ductal like structures. Previously, it was demonstrated that mTOR inhibition and Wnt activation is important for maintaining hematopoietic stem cells (Huang et al., 2012). However, in the case of salispheres, the dual treatments of LiCl and rapamycin affected their primary survival. Hence, this would strongly suggest that mTOR and Wnt are both important factors for *in vitro* culture of salispheres.

The exciting finding of adherent salispheres from ligated/de-ligated glands suggests that salisphere growth was altered by injury. The impairment of cytokeratins and protein expression of signalling pathways due to irradiation suggests that this might

be correlated to the morphological changes observed in salispheres derived from injured glands (Nanduri et al., 2013).

Since salispheres acquired a distinctive morphology, cytoskeletal rearrangements were initially suggested to be responsible for these changes. The second complex of mTOR and RhoA/ROCK signalling were selected to be investigated because they are both involved in cytoskeletal rearrangement. Morphological similarities, as well as the expression pattern of p-FAK and CK5 between ROCKi treated salispheres and grown salispheres from ligated glands, suggest minor alterations in ROCK signalling post-injury. While mTORC2 inhibition failed to show this phenotype. These data also imply that there are other factors behind the morphological alterations.

It has been reported that ROCK inhibition plays a role in the survival of human embryonic stem cells and decreases apoptosis (Watanabe et al., 2007; Li et al., 2009). In addition, ROCK inhibition may prevent salisphere senescence and enhance their expansion (Lee et al., 2015). Although, the proliferation of salispheres was stimulated by ROCKi, and similar to the study performed by Lee et al. the salispheres became adherent (cultured from un-operated gland). This suggests salisphere attachment is partly due to RhoA-ROCK signalling, and likely other unknown upstream signals. As ROCK inhibition showed some similarities to salispheres derived from injured glands, neural input may also play a role.

Although, *in vitro* and *in vivo* experiments of involving BoNT/A, neuropeptides and atropine suggest that BoNT/A was minimally related to the morphological alterations of salispheres, and other factors may be responsible for these changes. The role of nerves in stem/progenitor cells behaviour was highlighted by a recent paper, which

showed that salivary stem/progenitor cells were unable to regenerate post-irradiation due parasympathetic alterations (Knox et al., 2013). Atrophy caused by ductal ligation with or without including the chorda lingual nerve in rats (extraoral duct ligation and intraoral ligation respectively) is reversible (Scott et al., 1999; Osailan et al., 2006b; Carpenter et al., 2007), suggesting a minor influence. However, the neural influence is important for salivary secretion, as a recent study showed that inhibiting SP resulted in the atrophy of the acinar cells (Hishida et al., 2016). Presumably, the parasympathetic denervation mode of action during ligation is different compared to the mechanism of BoNT/A, which illustrates the differences between salispheres derived from injured glands and BoNT/A injected glands (Emmelin, 1968; Coskun et al., 2007; Proctor and Carpenter, 2007).

The stem/progenitor pool is localized in the ductal compartment. It is also presumed that if cell proliferation increases during injury, there would be no effect on the production of stem/progenitor cells *in vitro*. If regeneration could occur during ligation, the population of salispheres would not be affected as they represent the stem/progenitor cell pool. As regeneration models including salivary gland extirpation and ligation/de-ligation models have shown that extensive proliferation of ductal cells appear post-injury (Hanks and Chaudhry, 1971). However, the degree of obstruction is correlated with the level of ductal cell proliferation from residual and not actively proliferating ductal cells (Takahashi et al., 2000; Takahashi et al., 2004). In addition, it has been demonstrated that in the first period of ligation extensive ductal proliferation occurs, but at late period acinar cells apoptosis and fibrotic tissue are observed (Carpenter et al., 2007). Indeed, the reduction of salispheres post-injury could be apoptotic.

The protective role of mTOR within acinar cells could possibly imply that these acinar cells are involved in salispheres formation but not through acinar cell self-duplication (Silver et al., 2010; Aure et al., 2015). Another suggestion is that atrophy may drive these salispheres to differentiate as a consequence of the glandular activation of mTOR (Silver et al., 2010). In addition, the inhibitory effects of RhoA/ROCK could be correlated with differentiation, as observed in mouse neural stem cells (Gu et al., 2013). While, the remaining salispheres during ligation may be responsible for the asymmetric division during regeneration, as removal of the obstruction results in the recovery of the gland and salisphere population (Kimoto et al., 2008; Coppes et al., 2009). However, if progenitor cells undergo differentiation during atrophy, they may also undergo restrictive division driving differentiation (Coppes et al., 2009). The diagram below describes a possible mechanism based on stem/progenitor cells division during glandular atrophy:

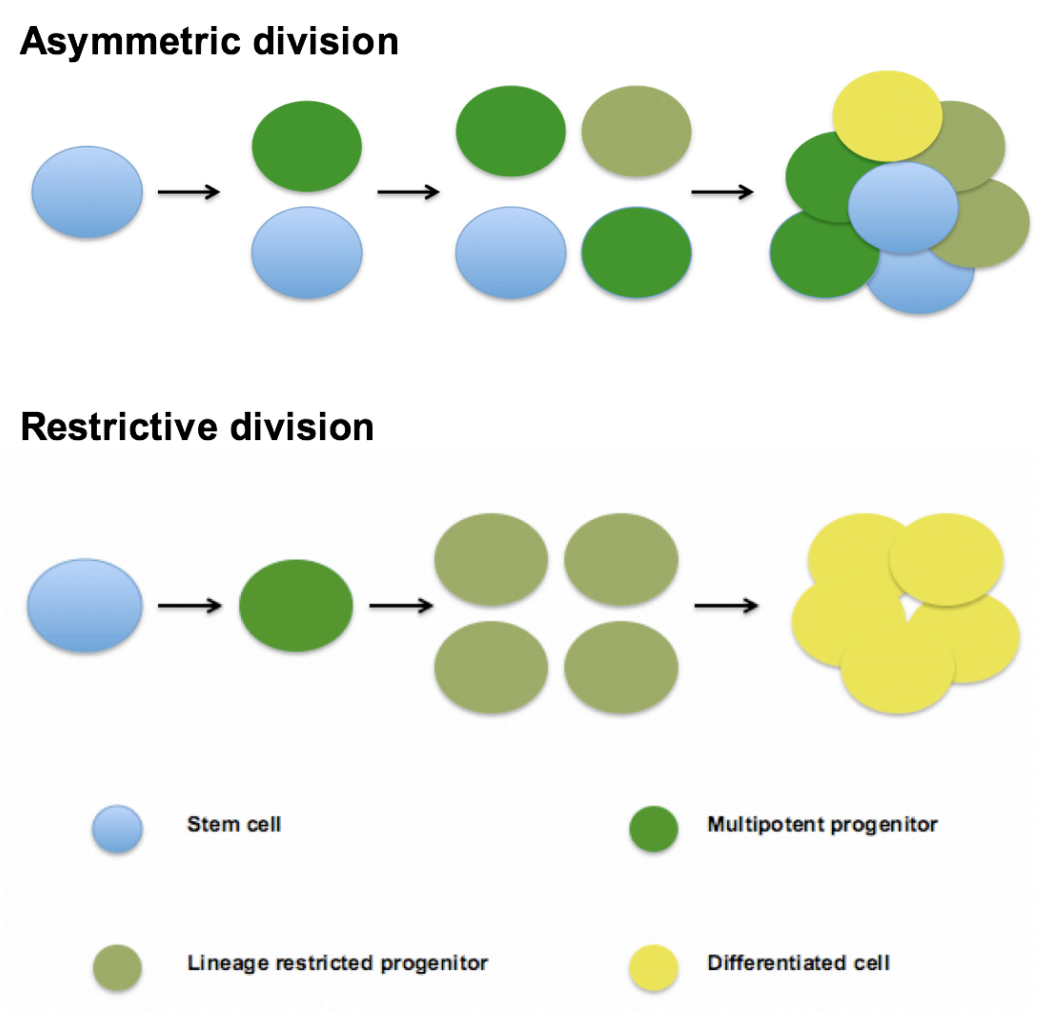


Figure 7.1 Asymmetric division and restrictive division. Asymmetric division is a process whereby a stem cell generates one daughter stem cell and one daughter multipotent progenitor cell. These two daughters produce a group of stem cells, multipotent progenitors and lineages restricted progenitors. Subsequently, these stem cells and progenitors are maintained, and are responsible for cellular differentiation. Restrictive division occurs in progenitor cells, which are more committed to producing differentiated cells only. Modified from Coppes et al., 2009.

Since several inhibitors such as rapamycin, LiCl and ROCKi have shown various effects on the culture of salispheres, intraglandular injections of treated salispheres with these inhibitors were applied. The *in vivo* transplantation of salispheres would translate the *in vitro* effects of the inhibitors on glandular recovery. Before examining the impact of salisphere transplantation on glandular recovery on injured glands, the injections were applied to controls to understand the physiological response of

normal glands to the injections. Although the intraglandular injections of salispheres into regenerated glands have been performed once, LiCl and ROCKi showed some effects either on glandular recovery or mTOR activation. The output of LiCl on salispheres culture could possibly responsible for glandular recovery of a regenerated gland. Indeed, administration of LiCl has been shown to accelerate the repair and recovery of renal function (Bao et al., 2014). This suggests that LiCl could play a role in enhancing the recovery process.

Salivary gland primary cell culture is heterogeneous (e.g parenchymal cells, neural cells), and expresses several stem cell markers (Coppes and Stokman, 2011). As the whole population will be transplanted it is difficult to distinguish which type of cells are responsible for the enlargement and recovery of the de-ligated gland. However, the *in vivo* effect of ROCK inhibition suggests that there is crosstalk between mTOR and ROCK. In addition, the activation of mTOR in salivary glands by ROCKi treated salispheres implies a connection between salivary gland atrophy and ROCK via mTOR signalling, as mTOR was activated during ligation (Silver et al., 2010).

In conclusion, mTOR is one of the important signalling networks for salispheres survival and development. The use of several inhibitors helped to identify several factors that may play a role in salisphere behaviour in healthy and injured glands. The exciting *in vitro* and *in vivo* effects of LiCl suggest that a GSK-3 inhibitor could stimulate salisphere growth and gland recovery presumably via Wnt and mTOR. In addition, the *in vivo* effects of ROCK inhibition suggest that ROCK signalling might be correlated to atrophy as it manipulated the characteristics of salispheres as well as glandular mTOR. However, further investigations are required to confirm these

findings, and to understand the molecular mechanism of intracellular signalling in salispheres during regeneration.

7.2 Future plan

Although the results achieved from this PhD have shown some novel insights of the involvement of mTOR in regeneration, they also pose more questions to answer for potential future work in this field.

- Transplantation of c-kit expressing cells into damaged glands has shown the ability of stem/progenitor cells to rescue salivary glands against damage (Lombaert et al., 2008a). Since *In vitro* experiments showed that LiCl has positively affected the culture of salispheres, it would be interesting to identify markers which are influenced by LiCl treatment.

- Since the culture of salispheres from ligated glands behaved differently compared to normal healthy salispheres, it would be interesting to determine if salivary gland stem/progenitor cells from injured glands are undergoing differentiation *in vitro*. In addition, injections of adherent salispheres into injured glands would help in investigating the regenerative capacity of these stem/progenitor cells, and in determining the relationship between the adherent property and regenerative potential.

- As ROCK inhibition showed some effects on mTOR activity and glandular mTOR is only activated during atrophy, glandular injections of ROCK inhibitor would help in answering if ROCK inhibition is associated with atrophy (Silver et al., 2010; Bozorgi et al., 2014). Further, culture of salispheres from injected normal glands with ROCKi would also assist in revealing whether RhoA/ROCK is impaired during ligation and responsible for alterations of salispheres (e.g. number, morphology) caused by ligation.

- Since the intraglandular injections showed variable effects on the total protein contents by Coomassie blue staining, it would be helpful to perform proteomic analysis, to explore which proteins are dysregulated post-injection.

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APPENDIX A

Injection of treated salispheres into a de-ligated gland	Gland weights (mg)
Untreated salispheres (Control inj)	45
LiCl treated salispheres (LiCl inj)	65.4
ROCKi treated salispheres (ROCKi inj)	47.4
Rapa+LiCl salispheres (rapa+LiCl)	57

Table 6.3 Gland weights of submandibular glands post-injection with untreated, LiCl, ROCKi and the co-treatment of Rapa+LiCl treated salispheres.

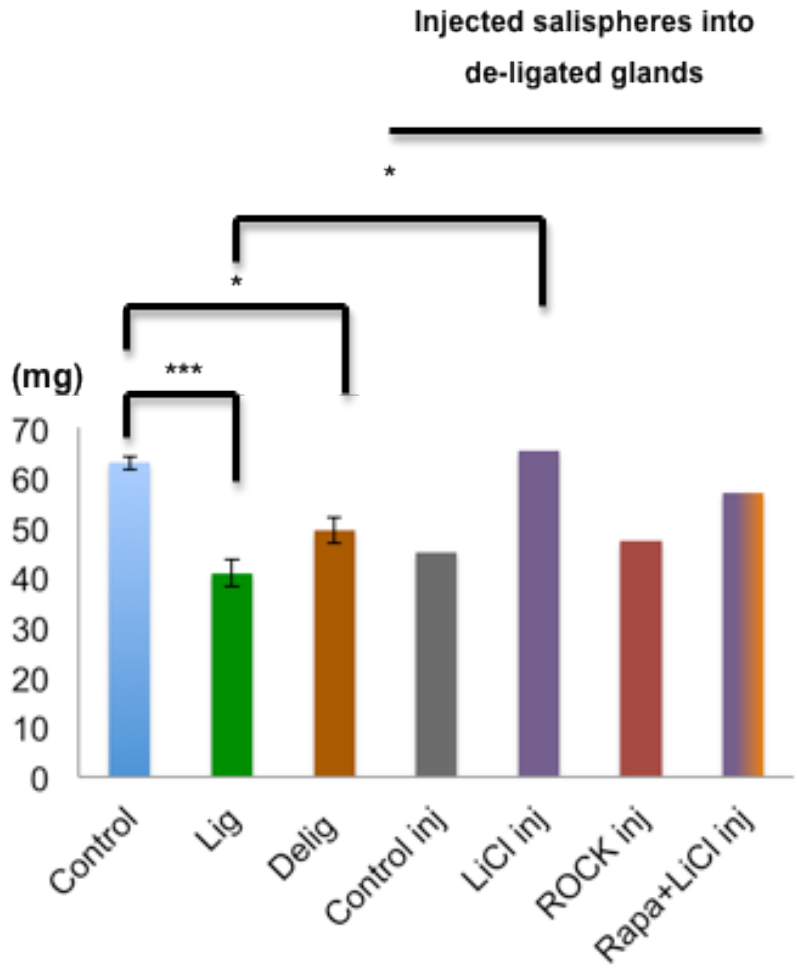

Figure 6.5 A graph representing the differences between weights of control, ligated, de-ligated and the gland weight post injection with untreated, LiCl, ROCKi and rapa+LiCl salispheres into regenerated gland. Ligation (p=0.0002) and de-ligation (p=0.014) caused a significant decline in gland weight. Injections of treated salispheres into regenerated glands showed that only LiCl treated salispheres significantly increased the gland mass, where the gland weight is approximately similar to un-operated gland.

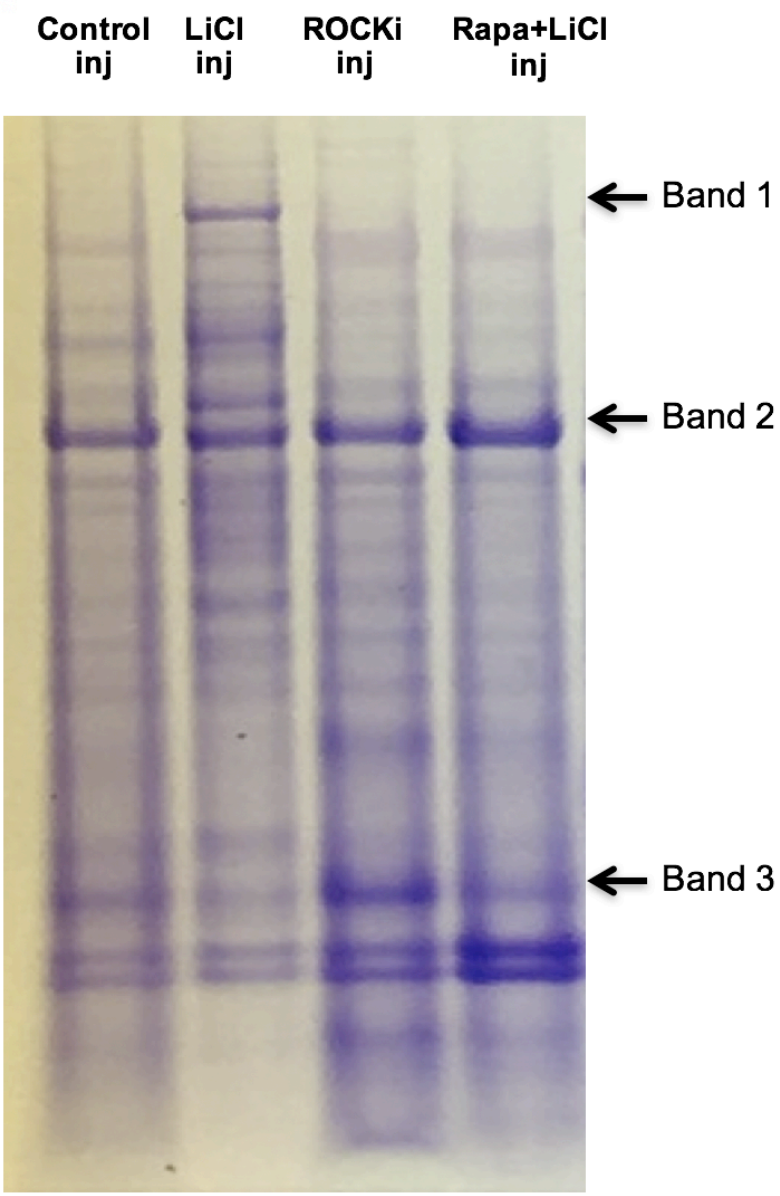

Figure 6.6 Coomassie blue staining of SDS-PAGE gels of de-ligated glands homogenates some variations in protein contents. All samples showed acinar mucins (band 2). However, the injections of LiCl treated salispheres and ROCKi treated salispheres showed distinctive banding pattern; band 1 and band 3 respectively.

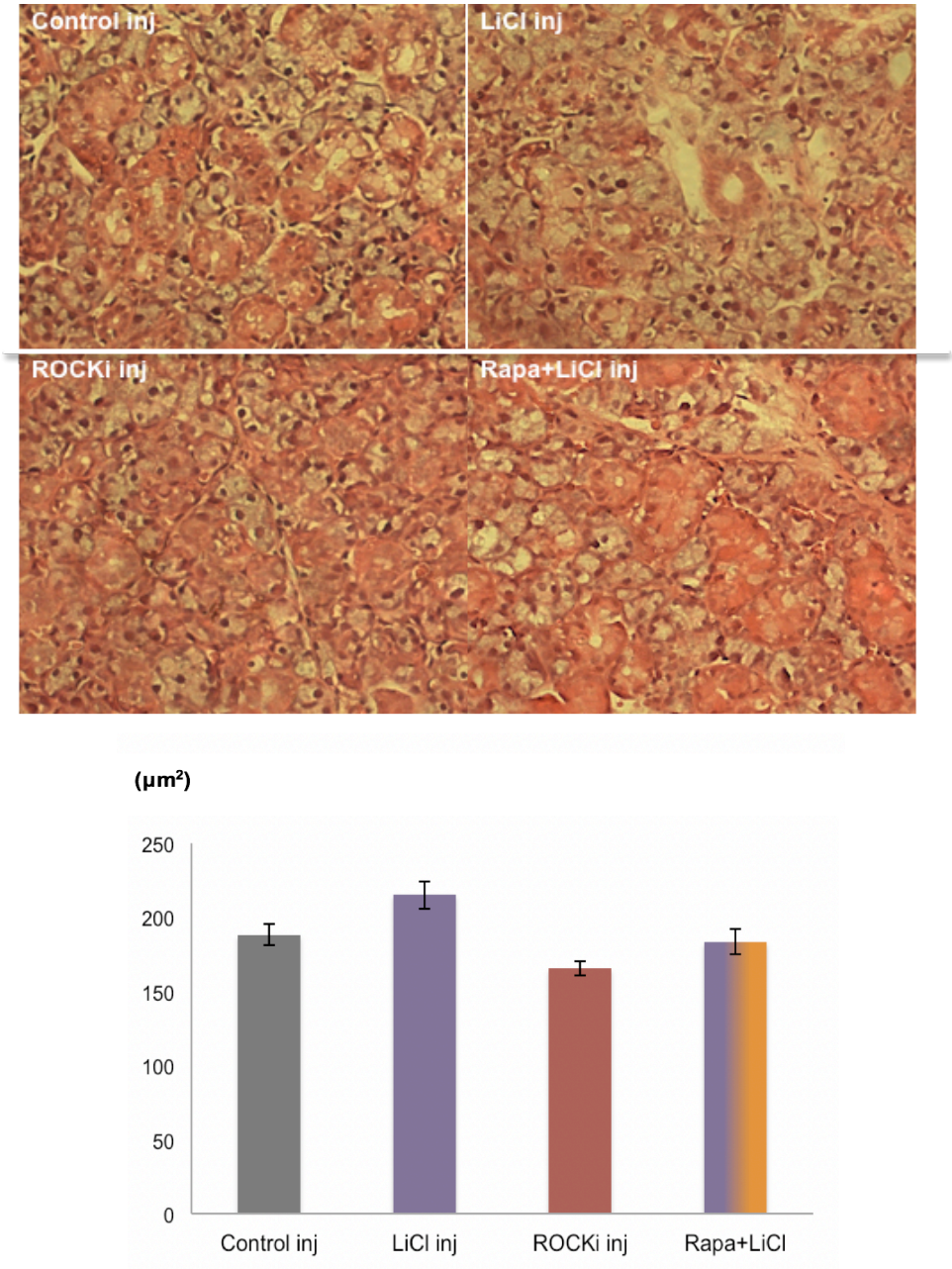

Figure 6.7 Histology and mean area size of acinar cells of regenerated glands post injection with untreated, LiCl, ROCKi and rapa+LiCl salispheres. Images of H&E staining illustrated normal histology of regenerated submandibular glands after injecting with salispheres. Acinar cell volume appeared approximately comparable among the four treatments, but a slight increase was observed in injected glands with LiCl treated salispheres.

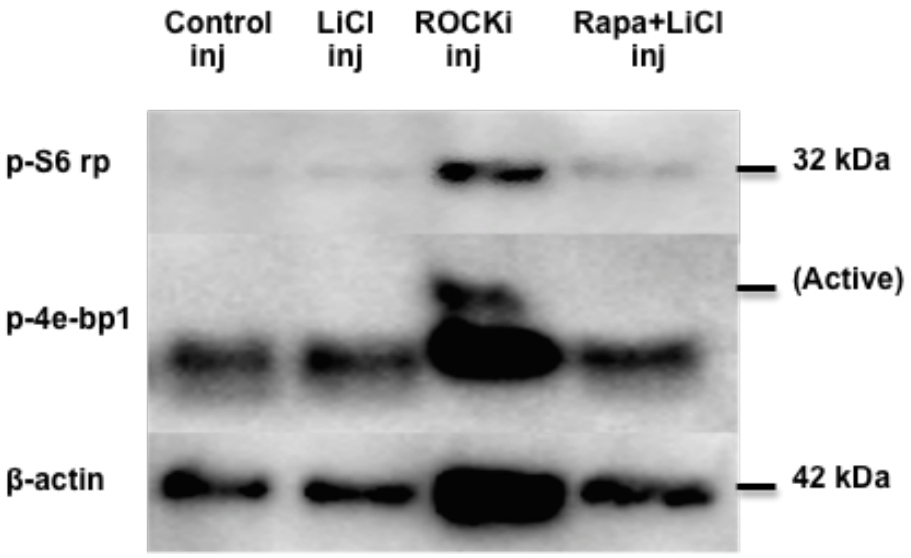

Figure 6.8 mTOR status post injections in de-ligated glands. All injections in regenerated glands appeared to phosphorylate 4e-bp1. However, ROCKi resulted in the phosphorylation of 4e-bp1 and the expression of S6 rp.

APPENDIX B

In Appendix B, a series of experiments using salivary gland tissue homogenates were used to illustrate the relationship between band intensity and exposure time, as shown in some immunoblots which appeared over exposed. This a supplementary section to justify using the ChemiDoc system on western blots. In addition, to compare the differences in protein expression between untreated samples and experimental samples, in arbitrary units.

The principle of the ChemiDoc imaging system avoids the limitations of x-ray film in recording chemiluminescent light, as the exposure time is limited when pixels become saturated. (red dots) Five dilutions of normal submandibular glands were used for gel electrophoresis and immunoblottting under reducing conditions. Table 2.2 below shows the concentrations of tissue homogenates:

Sample	Concentration (µg/ml)
C1	1000
C2	500
C3	250
C4	125
C5	62.5

Table 2.2 Concentrations of submandibular gland tissue homogenates.

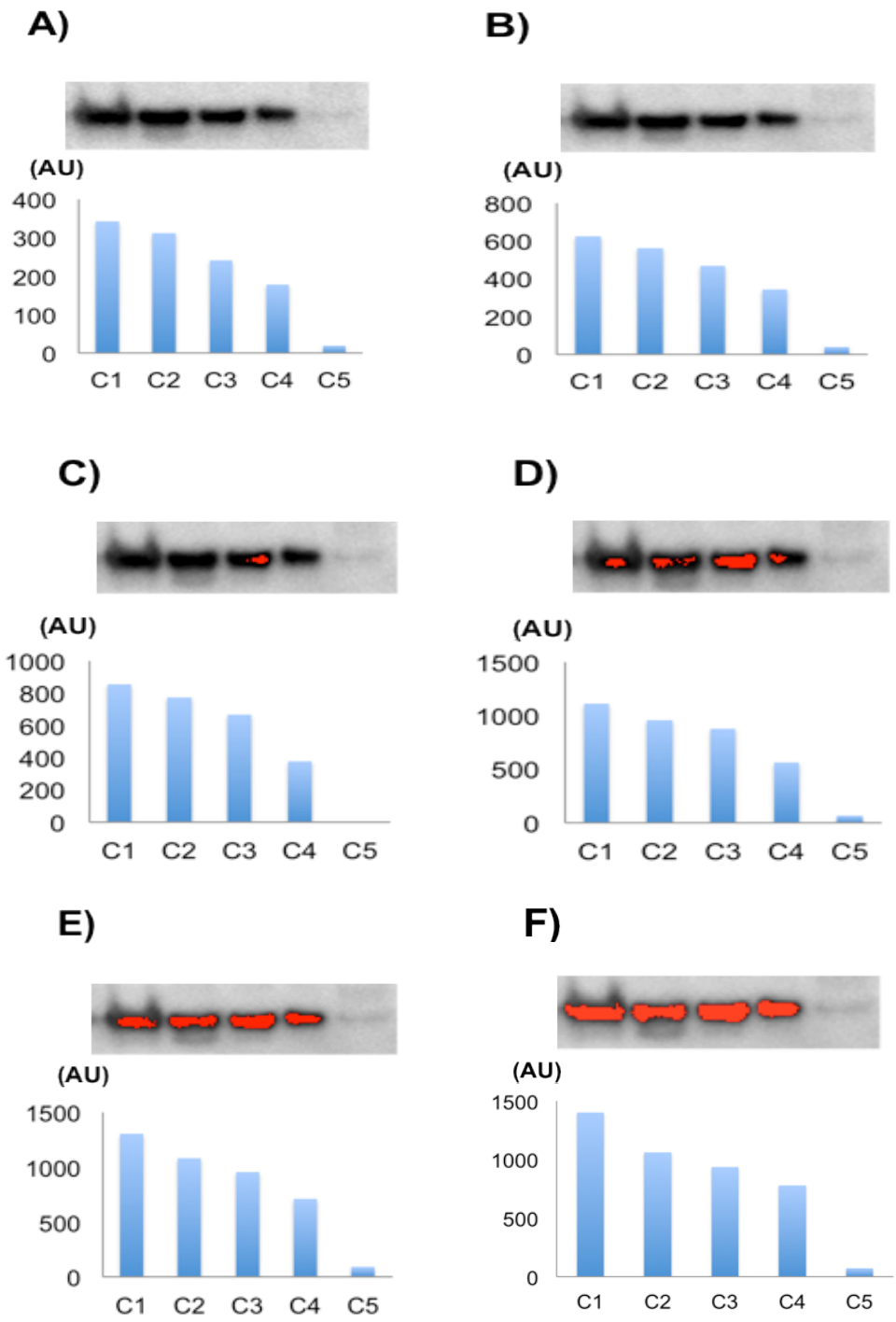

Figure 2.1 Immunoblots of β-actin band intensities in submandibular gland tissue homogenates at different time-points; A) 10 seconds, B) 20 seconds, C) 30 seconds, D) 40 seconds, E) 50 seconds and F) 60 seconds. The band intensities increase overtime and share similar banding pattern of C1, C2, C3, C4 and C5.

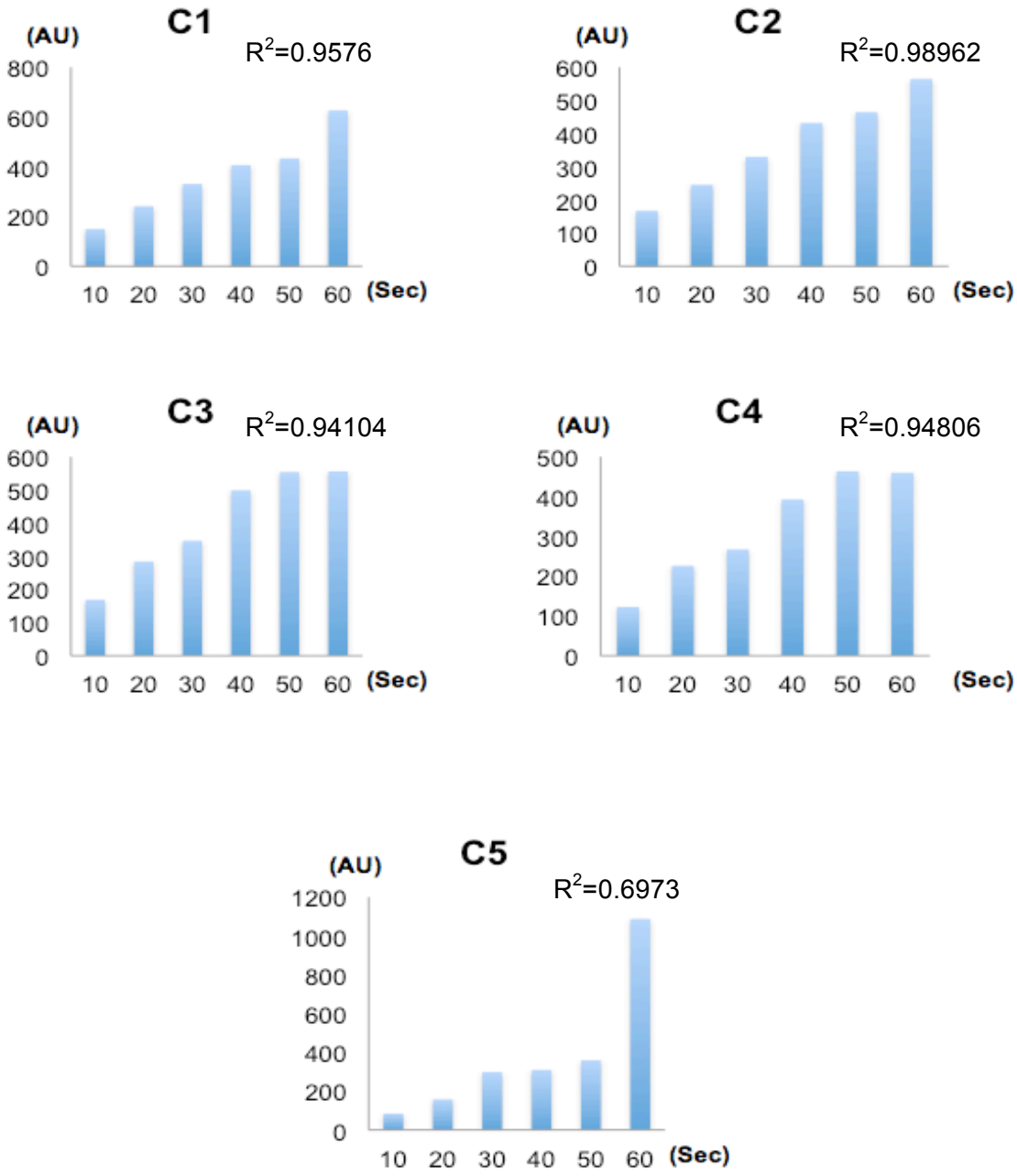

Figure 2.2 Band intensities of β-actin at different time points of five quantities of submandibular gland tissue homogenates. Charts represent five concentrations of submandibular gland tissue homogenates measured at different time. Band intensities as shown increased parallel to time of exposure in all samples (from the highest to the lowest protein concentrations of tissue homogenate).

The use of five quantities of submandibular glands tissue homogenates illustrated the parallel relationship between time of exposure and band intensity. The band intensities have been selected for all experiments around 10 and 30 seconds as intensities become saturated after 40 seconds.